Analysis of Adenosine 2a Receptors During the Immune Recognition of Foreign and Self-Antigens

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Dedication

To my Husband, you are my rock and I am your satellite

To my Father, who always nurtured my curious mind

To my Mother, who always nurtured my heart

To my Sister, who always nurtured my soul

To my Brother, who always nurtured my laughter

You are the pillars of my world

Abstract

Purinergic receptors are attractive therapeutic targets that can regulate several facets of the immune system. This dissertation examines the role of purinergic G protein-coupled Adenosine 2a (A2aR) receptors in regulating adaptive immune responses during the primary response to vaccination and during self-antigen recognition in the context autoimmunity. Administration of A2aR agonist CGS-21680 (CGS) can alter CD4 T cell differentiation by diverting helper T cells away from a germinal center follicular helper T cells (GC-Tfh) linage that promotes the survival, differentiation, isotype class-switching, and affinity maturation of B cells. Although GC-Tfh cells are beneficial during the clearance of certain pathogens, they also can be detrimental if they malfunction and mistakenly provide help to pathogenic self-specific B cells. Previous studies suggest that A2aRs acts as a barrier to autoimmunity by limiting inflammatory responses. Our studies support this claim by showing that prophylactic treatment with CGS blocks autoimmune arthritis. A2aR agonist treatment caused a reduction in the frequency and number of pathogenic GC-Tfh cells and isotype class-switched plasmablasts that respond to autoantigen. CGS treatment of mice *after* the early onset of mild arthritis also had a therapeutic benefit and blocked disease progression. CGS therapy reduced the number pathogenic GC-Tfh cells and autoantibody titers, suggesting that A2aR downstream signals may serve to limit dangerous GC-Tfh cell effectors that contribute to autoimmune disease manifestations, thus making it an attractive target for future immunotherapies.

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Chapter 1:

Background and Introduction

1.1 Purinergic receptors

Adenosine-5'-triphosphate (ATP) is multifaceted nucleoside that regulates several biological functions including immunoregulation (1,2). Under steady state conditions the concentration gradient of ATP is relatively higher in the cell cytoplasm versus the extracellular space. However, under stressful conditions (infection, tissue damage, or sterile inflammation) the extracellular milieu shifts toward a higher ATP concentration (2). ATP can be released from cell-membrane channels such as pannexins, connexins, maxi channels and P2X7R pores (3). Extracellular ATP (eATP) is considered a danger-associated-molecular pattern that can activate both innate and adaptive immune responses (3). Most immune cells express ATP specific inotropic receptors P2XRs that can promote the activation of monocytes, macrophages, dendritic cells, T cells, Regulatory T cells (Tregs), and invariant natural killer T (iNKT) cells (4). P2XRs can also elicit cytokine release of innate immune cells and IgE shedding by B cells (4).

The immunoreactive effects of eATP can be counter-regulated by ectoenzymes CD39 (ENTPD1) and CD73 (ecto-5'-nucleotidase) that sequentially hydrolyze ATP to AMP (adenosine 5'-monophosphate) by CD39 and AMP to extracellular adenosine by CD73 (5,6,2). Adenosine is considered an immunosuppressive purine nucleoside that governs several inflammatory responses via four different G protein–coupled adenosine receptors (A1, A2a, A2b, A3) (7,8). Adenosine A2a receptors (A2aRs) are expressed primarily on cells of hematopoietic origin, particularly on activated cytotoxic CD8 and helper CD4 T cells (9–11). Studies using mouse models of T cell–mediated autoimmune

disorders (12, 13) as well as graft-versus-host disease (14) have shown that A2aR signaling can restore immune homeostasis by promoting the induction of T cell anergy, regulatory T cell (Treg) differentiation, and suppression of effector T cell functions.

1.2 Immunomodulatory effects of A2aR signals

A. Anergy

Anergy is a mechanism of peripheral immune tolerance that drives potentially dangerous T cells toward a state of functional unresponsiveness that is characterized by defective IL-2 production, inhibited cell cycle progression, and obstructed effector/memory T cell differentiation (15). Previous studies report that endogenous adenosine and selective A2aR agonists can inhibit dangerous effector responses to self-antigen by promoting a state of functional unresponsiveness in autoreactive self-specific T cells (12). Powell and colleagues offered a mechanistic explanation of how A2aR signals promote anergy by inhibiting IL-2 production (18). A2aR activation enhances intrinsic levels of cAMP (Cyclic adenosine monophosphate), a second messenger that can bind to and activate downstream molecules such as the cAMP-dependent kinase, protein kinase A (PKA). Enzymatically activate PKA phosphorylates transcription factors like cAMP responsive element-binding protein (CREB) that sit either in proximity or directly on the promoter regions that drive the transcription of A2aR-regulated genes (16, 17) (Fig. 1). Phosphorylation of CREB (p-CREB) at the conserved serine residue 133 can promote the recruitment of the transcriptional co-activator

cAMP-responsive element modulator (CREMα) (17-19). CREB and CREMα can form a heterodimer complex that binds upstream of the IL-2 promoter-enhancer site and suppress the production of IL-2 (18). Therefore, it is possible that A2aR promotes T cell anergy by reducing IL-2.

B. Regulatory T cells

Regulatory T cells (Tregs) have a high co-expression of cell-surface ectoenzymes CD39 and CD73 that breakdown extracellular ATP to adenosine (Fig. 2)(49). Extracellular adenosine can enhance the immunoregulatory activity of Tregs by activating A2a receptor (A2aR) signals that enhance their suppressive functions (12, 49, 50). Studies using mouse models of colitis and graft versus host disease have shown diminished protection in mice with A2aR-deficient Tregs (13,51) (Fig. 2). Ohta et al. (52) reported that in addition to enhancing Treg suppression, A2aR signals can also enhance the proliferation of natural Tregs and promote the induction of new Treg (Fig. 2). A study by Zarek et al. supports these observations by reporting enhanced Foxp3 and LAG3 mRNA levels in T cells activated in the presence of a selective A2aR agonist (12). A2aR activation can also enhance the production of TGF-β, an essential molecule for the induction of Tregs (12). Therefore, it is possible that adenosine promotes T cell tolerance by activating A2aR signals that enhance the performance of Tregs and increase the number of Tregs by both inducing new Tregs and expanding natural Tregs.

C. Effector T cells

A2aRs negatively regulate adaptive immune responses by inhibiting the effector functions of lymphocytes (2,7). Loss of A2aR signaling in *Adora2a*-deficient (A2aR KO) mice leads to enhanced control of tumor growth by CD8 T cells (20). An investigation of tumor Ag–specific CD8 polyclonal T cells in A2aR KO mice revealed that endogenous adenosine limits their clonal expansion and effector functions needed to effectively eradicate tumors (20). *In vitro* polarizing assays suggest that A2aR signaling alters CD4 T cell differentiation by inhibiting the induction of Th1 ($Tbet^+$), Th2 ($GATA3^+$), and Th17 ($RORgt^+$) effector T cell phenotypes (21, 22). *In vivo*, activation of A2aRs during acute infection with *Toxoplasma gondii* reduced the number of Th1 IFN-γ–producing CD4 T cells (24). In a mouse model of asthma, selective A2aR agonists also reduced the expression of RORγt, the master regulator of Th17 cells, in the lung tissue of affected mice (23). This matched the reduced production levels of IL-17 in affected mice (23). Models of colitis, graft-versus-host disease (GVHD), and autoimmunity also observed enhanced survival due to a decrease production of IL‑6, TNFα and IFNγ suggesting that A2aR signaling can reduce Th1 and Th17 responses (4). However, until very recently no studies had indicated a role for A2aR signaling in Tfh differentiation despite the role of other purinergic receptors such as P2X7 in regulating Tfh homeostasis (25). It is possible that A2aR-mediated effects on Tfh cells may have been overlooked due to a requirement of Ag specificity between T cells and B cells during Tfh differentiation (26).

C. Humoral immune responses during hypoxia

Abbott and colleagues reported that the germinal center (GC), where B cells undergo class-switch recombination, somatic hypermutation, and clonal selection, develop an oxygen-low hypoxic microenvironment (53). Hypoxia creates an extracellular adenosine rich environment by releasing extracellular adenine nucleotides ATP and AMP into the extracellular matrix that can be broken down to adenosine by purinergic receptors CD73 and CD39 (54). Interestingly, hypoxia-inducible factor-1α (HIF-1α) can promote the transcription of CD73 and CD39 (55). Metabolically stressed cells from hypoxic conditions can also release extracellular adenosine via nucleoside transporters (56). However, excess extracellular adenosine is quickly eliminated by mechanisms that regulate extracellular adenosine levels by promoting the uptake of adenosine or by enhancing the cell surface expression of adenosine deaminase (ADA), a molecule that break adenosine to inosine (57, 58). Hypoxic environments can diminish the enzymatic activity of both adenosine kinases and ADA, therefore hypoxia not only promotes the production of extracellular adenosine, but also inhibits the mechanisms that clear excess extracellular adenosine. HIF-1α can also enhance the transcription of A2a and A2b receptors (59), therefore it is plausible that adenosine may be acting on a variety of immune cells in a hypoxic germinal center that regulates antibody mediated immune responses.

1.3 Germinal Center Follicular Helper T cell differentiation and function

Follicular helper (Tfh) differentiation involves the interplay of T cell intrinsic and extrinsic factors that push an uncommitted CD4 T cell progenitor toward a Tfh lineage (26). T cell extrinsic signals are exchanged between CD4 T cells and dendritic cells (DCs) through costimulatory receptor/ligand interactions such as CD28/(CD80/CD86) and inducible T cell costimulator (ICOS)/ICOS ligand (26,28). Cross talk between T cells and DCs can also occur through the secretion and recognition of effector cytokines like IL-6 and IL-21(26). These T cell extrinsic signals work in synergy to initiate the transcription of Tfh promoting factors such as B-cell lymphoma 6 protein (Bcl-6) (29, 30, 31), Maf (26, 27, 31), and signal transducer and activator of transcription 3 (STAT3) (26, 27, 31) while at the same time suppressing Tfh repressing factors such as PR domain zinc finger protein 1 (BLIMP-1) (29, 32, 33), forkhead box proteins FoxP1 (33, 34), Foxo1(35, 33) and STAT5 (31, 33). Tfh differentiation also requires the expression of chemokine receptor 5 (CXCR5) that allows CD4 T cells to migrate toward the B cell follicle (26). At the B cell follicles, CD4 T cell progenitors exchange signals with B cell that allow them to enter the specialized lymphatic structure known as a germinal center (GC) (30, 37). Once in the GC, CD4 T cell progenitors take on a GC-Tfh phenotype by expressing effector molecules like CD40L (31, 37) as well as signaling lymphocytic activation molecule (SLAM) associated receptors (29, 32) and programed cell death (PD-1) receptor (29,36). Furthermore, GC-Tfh cells also secrete effector cytokines such as IL-21 and IL-4 that promote the propagation and class-switching of B cells (38).

1.4 Discussion

Helper CD4 T cells are critical regulators of adaptive and innate immune responses. Their absence is drastically emphasized in patients with acquired immune deficiency syndrome (AIDS) or severe combined immunodeficiency (SCID) who are unable to clear infections caused by otherwise harmless microbes. CD4 T cells carry out a broad-spectrum of effector functions uniquely suited to eliminate specific pathogens by activating macrophages, cytotoxic T CD8 cells, and B cells. Identifying the factors that fine-tune CD4 T cell responses may be instrumental in the development of immune-targeted therapies and new vaccine approaches.

Although CD4 T cells are essential for immune homeostasis, they can at times malfunction, confuse self-antigens for foreign invaders, and mount inappropriate responses against self-tissues. Enhanced frequencies of GC-Tfh cells have been reported in patients suffering from antibody-mediated autoimmune disorders such as Rheumatoid Arthritis (RA) and systemic lupus erythematosus (SLE) (43,45). Multiple studies have shown a tight correlation between the frequency of GC-Tfh cells and the onset of disease (44).

Autoimmune disorders affect approximately 50 million Americans and are among the top 10 causes of death for women under the age of 65 (47). While many therapies are effective at alleviating pain and inflammation, most are not curative and do little to stop or reverse disease progression (47). Understanding the mechanisms that drive and sustain tolerance in healthy individuals may unlock the key to curative therapies that may go beyond pain alleviation and restore immune homeostasis.

The therapeutic benefits of adenosine in autoimmune disorders have been previously reported in patients treated with methotrexate (48) and sulfasalazine (60), popular disease-modifying antirheumatic drugs (DMARD) commonly prescribed to individuals suffering from systemic autoimmune disorders like RA. The anti-inflammatory effects of methotrexate and sulfasalazine are attributed to augmented levels of extracellular adenosine (60). Therefore, identifying the factors downstream of adenosine that alleviate symptoms of arthritis may create a unique opportunity to generate more effective next-generation immune targeted therapies to treat individuals suffering from autoimmune disorders.

Chapter 2:

Activation of A2aRs during vaccination

2.1 Introduction

Adenosine A2a receptor (A2aR) signaling acts as a barrier to autoimmunity by promoting anergy, inducing regulatory T cells, and inhibiting effector T cells. However, *in vivo* effects of A2aR signaling on polyclonal CD4 T cells during a primary response to foreign antigen (Ag) has yet to be determined. To investigate the *in vivo* effects of A2aR signaling on naïve polyclonal CD4 T cells during a primary response to foreign Ag we used a vaccination approach that allowed us to track Ag-specific CD4 T cells that differentiate into various T cell lineages such as Th1, Th17, Tregs, and T follicular helper (Tfh) cells. No reports to date have indicated a role for A2aR signaling in Tfh differentiation despite the role of other purinergic receptors such as P2X7 in regulating Tfh homeostasis (25). It is possible that A2aR-mediated effects on Tfh cells may have been overlooked due to a requirement of Ag specificity between T cells and B cells during Tfh differentiation (26).

To address this, we used a vaccine that consists of 2W1S peptide covalently coupled to PE (61). This allowed us to look at the interplay between endogenous Ag-specific 2W1S: I-A^b-specific T cells and PE-specific B cells (61). We initially sought to characterize polyclonal Ag-specific CD4 T cell anergy induction and Treg generation during A2aR signaling by tracking of 2W1S: I-A^b tetramer-binding T cells in mice treated with the selective A2aR agonist CGS-21680 (CGS). We discovered that CGS has no impact on the Ag-induced clonal expansion of polyclonal 2W1S-specific CD4 T cells, nor does it promote the induction of anergy or Treg differentiation in our vaccination system. CGS did

not appear to reduce Th1 or Th17 differentiation; instead, A2aR signaling directly inhibited 2W1S: I-A^b-driven CD4 T cell differentiation toward the germinal center (GC)-Tfh fate, and reduced cognate GC B cell responses. Therefore, this work identifies a novel A2aR-mediated control mechanism during vaccination that regulates CD4 T cell differentiation and function

2.2 Activation of A2a receptors during the primary response to vaccination fails to induce anergy or promote the differentiation of Tregs

The activation of A2aRs in many biological systems is associated with the production of intracellular cAMP, a second-messenger known for its anti-proliferative function (62). To investigate the effects of A2aR signaling during the primary CD4 T cell response to antigen, we used a tetramer of the major histocompatibility complex II I-A^b molecule containing the 2W1S peptide to study the *in vivo* proliferation and differentiation of polyclonal 2W1S: I-A^b-specific CD4 T cells following immunization with a 2W1S peptide coupled to PE in CFA. This vaccination approach induces 2W1S: I-A^b-specific CD4 T cells to undergo clonal expansion and differentiation to Th1, Th17, Tregs, and Tfh lineages (61). Coupling 2W1S and PE together also allows for the interplay between 2W1S-specific GC-Tfh cells and PE-reactive GC B cell. This occurs when PE-specific B cells internalize 2W1S-PE through BCR recognition of PE resulting in the display 2W1S: I-A^b complexes, and allows them to exchange helper signals with 2W1S: I-A^b–specific CD4 T cells (61). The effects of A2aR activation were tested by treating immunized mice twice daily with the selective

A2aR agonist CGS (2.5 mg/kg i.p.) or vehicle alone as a control (PBS) (12). As shown in figure 3A, the clonal expansion of the 2W1S: I-A^b-specific polyclonal CD4 T cell population as a whole was no different in CGS- or PBS-treated mice. Consistent with preserved clonal expansion, we also observed no increase in the number of 2W1S: I-A^b-specific Foxp3$^+$ Tregs during this immunization in the presence of CGS (Fig. 3B). Therefore, A2aR signaling did not inhibit clonal expansion nor did it enhance Treg cell differentiation after vaccination with antigen in CFA.

We next examined the capacity of A2aR signaling to promote anergy in 2W1S: I-A^b-specific polyclonal CD4 T cells during vaccination. To test the effects of A2aR signaling on the development of anergy, we examined two surface molecules whose high gene expression marks functionally unresponsive anergic T cells: CD73 (*Nt5e*) and FR4 (*Izumo1r*) (63, 64). Remarkably, CGS treatment led to a reduction rather than rise in the number of 2W1S-specific CD4 T cells that expressed high levels of these two anergy markers (Fig. 3C). Also consistent with an inability to promote tolerance in this system, CGS treatment led to an increase in the fraction of 2W1S: I-A^b-specific CD4 T cells that continued to express high levels of the proliferative marker Ki67 at day 7 (Fig. 3D). Taken together, these data indicated that A2aR signaling cannot inhibit the proliferation of polyclonal antigen-specific CD4 T cells during antigen priming in the presence of a strong adjuvant, and does not promote Treg generation or anergy induction.

2.3 Activation of adenosine A2a receptors interferes with the differentiation of Tfh and GC-Tfh cells during antigen priming

Paradoxically, primed CD4 T cells in CGS-treated mice expressed even lower levels of the anergy markers CD73 and FR4 than mice exposed to vehicle alone (Fig. 3C). Previous studies have demonstrated moderate levels of FR4 and CD73 expressed on Tfh cells (65). Although the role of these two molecules in Tfh generation and function remains unknown, this observation led us to the hypothesis that A2aR signaling interferes with Tfh differentiation. Mice primed with 2W1S in CFA did develop a large population of $CXCR5^{+}$ 2W1S: $I\text{-}A^{b}$-specific CD4 T cells that expressed moderate levels of FR4 and CD73 (Fig. 2 A, B). In contrast, treatment of primed mice with CGS led to a loss in the generation of this $FR4^{int}$ $CD73^{int}$ $CXCR5^{+}$ 2W1S: $I\text{-}A^{b}$-specific CD4 T cell subset.

To more formally assess A2aR-regulated Tfh differentiation, we investigated the expression of the cell surface marker PD-1 and the transcription factor Bcl6 in primed 2W1S: $I\text{-}A^{b}$-specific CD4 T cells (31, 32). CD4 T cells that express the highest levels of CXCR5, Bcl6, and PD-1 have been characterized as GC-Tfh cells ($Bcl6^{hi}$ $CXCR5^{hi}$) and are known to provide cognate help to antigen-specific B cells within germinal centers, whereas CD4 T cells expressing lower levels are characterized as Tfh cells ($Bcl6^{lo}$ $CXCR5^{lo}$) and reside at the T cell/B cell border (66). During primary immunization, CGS treatment reduced both the frequency and number of 2W1S: $I\text{-}A^{b}$-specific Tfh and GC-Tfh cells (Fig. 4 C-E). Consistent with a shift toward alternate differentiation fates, CGS treatment also elicited a small but significant increase in the number of 2W1S: $I\text{-}A^{b}$-specific non-

Tfh cells. Therefore, our experiments revealed a novel role for A2aR pathway activation in the inhibition of Tfh and GC-Tfh cell differentiation.

2.4 A2aR inhibition of Tfh differentiation is T cell intrinsic

Although A2aR expression is known to be highly induced on CD4 T cells following TCR ligation (9-11), the expression of this adenosine receptor might also be expected on other cells of hematopoietic origin. Additionally, Tfh cell differentiation is a multifactorial process whose regulation likely involves multiple additional cell types, particularly dendritic cells and B cells (31, 32, 66). Therefore, it was important to determine whether the effects of CGS were the result of direct A2aR engagement on the 2W1S: I-A^b-specific CD4 T cells. To address this question, CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (KO) mice lacking A2aRs only on their T cells were immunized with 2W1S-PE and compared to wildtype (WT) A2aR-expressing littermates following a 7d course of CGS treatment. In the absence of T cell-expressed A2a receptors, CGS treatment lost its capacity to reduce Bcl6 expression and block 2W1S: I-A^b-specific GC-Tfh differentiation, and its inhibitory effects on Tfh cells appeared greatly blunted (Fig. 5 A, B). Likewise, treatment of CD4-Cre *Adora2a*$^{f/f}$ mice with CGS failed to induce an increase in non-Tfh cells during 2W1S antigen priming. Given that Bcl6 promotes differentiation to the Tfh and GC-Tfh fates in part by repressing other lineage-specific transcription factors such as Tbet and RORγt (31, 32, 66), we further assessed these non-Tfh cells. WT mice significantly increased the frequency and number of 2W1S: I-A^b-specific RORγt$^+$ Th17 cells when their

A2aRs were directly bound by the adenosine agonist, whereas Tbet^{+} Th1 and Foxp3^{+} Treg differentiation appeared not to be regulated by A2aR signaling (Fig. 5 C-E). A small, but statistically insignificant increase in RORγt $^{+}$ Th17 cells was also observed in KO mice treated with CGS (Fig. 5 C-E). Thus, direct CD4 T cell A2aR signaling shifts the balance of differentiation away from the GC-Tfh fate toward Th17 effector cell generation during the primary response to antigen.

2.5 T cell-intrinsic A2aR activation reduces T-dependent B cell immunity

GC-Tfh cells promote the survival, differentiation, isotype class switch, and affinity maturation of antigen-specific B cells in germinal centers (66). Therefore, we hypothesized that A2aR signaling in T cells during primary immunization would interfere with the T-dependent B cell response to vaccination. To test this, WT and CD4-Cre *Adora2a*$^{f/f}$ mice (KO) mice were immunized with a protein complex containing 2W1S coupled to PE (2W1S-PE) either with or without CGS treatment, and then PE-specific B cells were enriched using magnetic beads and characterized by flow cytometry. An ~50-fold expansion of PE-specific B cells was seen in WT and KO PBS-treated mice following immunization, as compared to naïve mice (Fig. 6 A-C). Consistent with the hypothesis, the number of PE-specific B cells found after vaccination was significantly reduced in CGS-treated WT hosts, but not in mice whose CD4 T cells lacked A2aRs. CGS treatment appeared to have its greatest inhibitory effect on the frequency and number of GC-phenotype CD38^{-} GL7^{+} PE-binding B cells in the WT mice, although all B cell subpopulations were affected (Fig. 6 A-D).

Importantly, these inhibitory effects of CGS on PE-specific B cells during immunization were blunted in mice that lacked *Adora2a* gene expression specifically within the T cell compartment. Therefore, these data suggest that the loss of antigen-specific GC-Tfh differentiation that occurs during strong A2aR signaling on CD4 T cells is sufficient to abrogate the provision of cognate T cell help to GC B cells during their primary response to antigen.

2.6 A2aR signals reduce the number of germinal centers

WT and KO hosts were immunized with 2W1S-PE and treated with PBS or CGS. Tissue slides of whole spleen images were collected and germinal centers were addressed by staining for GL7, IgD, B220, and Dapi. $GL7^{+}B220^{+}Dapi^{+}IgD^{-}$ germinal centers were manually quantified (Fig. 7A). The frequency of the germinal centers was elevated in both WT and KO immunized mice compared with unimmunized controls (Fig. 7B). CGS treatment in WT immunized mice reduced the frequency of germinal centers compared to PBS treated WT immunized mice (Fig. 7B). This matches our flow cytometry data where we observed a lower frequency of GC-Tfh and GC- B cells in WT mice treated with CGS. The frequency of germinal centers in KO immunized mice treated with CGS was comparable to PBS treated WT and KO groups (Fig. 7B). This also matched our flow cytometry observations and suggested that A2aR disruption of germinal centers is T-cell dependent. In addition, we also zoomed in on specific germinal centers and saw that the few germinal centers found in unimmunized mice appeared smaller in size compared to immunized WT and KO mice. Agonist

treatment seems to slightly decrease the GC size in WT mice; however, the size of the GCs in KO mice treated with CGS appears comparable to KO PBS treated. mice (data not shown).

2.7 Discussion

The identification of novel signaling elements that fine-tune CD4 T cell lineage differentiation during primary immunization offers new opportunities to improve the efficacy of vaccines and targeted immunotherapies (36, 67, 68). Our data suggest that *in vivo* A2aR signaling during the primary response to antigen plus a strong adjuvant diverts CD4 T helper cells away from the Tfh and GC-Tfh lineages. Given the key role of alternative T differentiation fates such as Th17 in protection against mucosal barrier infections (36), it is conceivable that selective A2aR agonists could be useful during vaccination to shape an optimal T differentiation response against pathogens.

A2aRs also appear to be a particularly attractive therapeutic target for the treatment of B cell-dependent autoimmune disorders such as systemic lupus erythematosus (45,69) and rheumatoid arthritis (RA) (43,44). Indeed, previous studies have shown a positive correlation between the number of Tfh cells and disease burden in patients with RA (43,44). Perhaps consistent with this, A2aR agonists effectively suppress animal models of inflammatory arthritis (70). The fact that the first-line anti-rheumatic drugs methotrexate and sulfasalazine act, in part, through the generation of extracellular adenosine and A2aR signaling (60), lends further support to the notion that A2aR signaling can ameliorate T-

dependent B cell autoreactivity.

It should be noted that the ablation of A2aRs in these vaccination experiments using conditional knock-out mice failed to significantly enhance antigen-specific Tfh or GC-Tfh differentiation. Similarly, use of a selective antagonist of A2aRs during immunization did not reliably alter CD4 T cell differentiation (data not shown). Although strong A2aR signaling with an agonist can be inhibitory for Tfh and GC-Tfh differentiation in normal CD4 T cells, under normal circumstances endogenous extracellular adenosine may play no role in CD4 T cell fate selection within secondary lymphoid organs following i.p. immunization in adjuvant. Alternatively, unspecified factors (e.g., increased A2bRs, decreased adenosine kinase) may compensate for the loss of A2aRs in KO mice. Activated T cells are known to upregulate adenosine deaminase (ADA), an enzyme capable of metabolizing adenosine to inosine (70). Therefore, strong continuous A2aR signaling from endogenous adenosine sources may only occur under special circumstances such as hypoxia where CD73 is up-regulated to facilitate increased extracellular adenosine production in close proximity to A2a receptors (59, 72). Going forward, it will be important to identify the immunological context (spatial and temporal) whereby extracellular adenosine counter-regulates Tfh and GC-Tfh differentiation.

3.8 Materials and Methods:

Mice

B6 (WT) mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD). *Adora2a*$^{f/f}$ mice containing *loxP* sites on either side of exon 2 of the *Adora2a* gene (a gift from Joel Linden, La Jolla Institute for Allergy and Immunology, La Jolla, CA) (11) were crossed with CD4-Cre mice (a gift from Michael Farrar, University of Minnesota, Minneapolis, MN) to generate conditional A2aR T cell knockout (KO) mice. Non-Cre littermates were used as WT controls. Mice were bred and housed in specific-pathogen free conditions in animal facilities at the University of Minnesota, Twin Cities. All experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and the National Institutes of Health.

Immunization and selective A2aR agonist treatment

Mice were given an intraperitoneal (i.p.) vaccine containing 200 μl of 0.6 μg of 2W1S peptide conjugated to 25 μg of PE emulsified in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich) as previously described (61). Mice were then given a 7d course of twice daily i.p. injection with the selective A2aR agonist, CGS-21680 (CGS; Tocris) 2.5 mg/kg or with vehicle alone (PBS) as previously described (12).

Cell enrichment and flow cytometry

Lymph nodes (LN) and spleens were collected and divided for separate enrichments of 2W1S:I-A^b tetramer-specific CD4 T cells and PE-specific B cells. 2W1S:I-A^b APC-labeled tetramers were used to stain and enrich for 2W1S-specific CD4 T cells (61). PE B cell enrichment was performed by mixing cell suspensions with 1 μg of PE (ProZyme) (61). Isolation of PE-specific B cells and for 2W1S: I-A^b-specific CD4 T cells was done using magnetic beads (STEM cell Technologies) (61). Enriched 2W1S T cells were first surface stained with CXCR5 (2G8), PD-1 (J43), CD4 (RM4-5), CD44 (IM7), as well as with the irrelevant cell exclusion antibodies CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8), and then fixed/permeabilized using a fixation/permeabilization kit (eBioscience) followed by intracellular staining with Foxp3 (FJK-16s), Tbet (4B10), Bcl6 (K112-91), RORγ (Q31-378), and Ki67 (SoA15). Enriched PE-specific B cells were surface stained with B220 (RA3-6B2), GL7 (GL-7), CD38 (90), IgM (RMM-1), and IgD (11-26c.2a), as well as with the irrelevant cell exclusion antibodies CD11c (N418), CD4 (GK1.5), CD8 (53-6.7), and F4/80(BM8), and then they were fixed/permeabilized using a fixation/permeabilization kit (eBioscience) and intracellular stained with goat anti-mouse Ig (H+L) (A11068). Anergy in 2W1S:I-A^b-specific CD4 T cells T cells was assessed by staining with CD73 (TY11.8) and folate receptor 4 (FR4, 12A5) as previously outlined (63, 64). Cells were analyzed on a Fortessa (Becton Dickinson) flow cytometer and analyzed using FlowJo (TreeStar).

Immunohistology

Spleens from WT and KO hosts immunized with 2W1S-PE and treated with PBS or CGS were frozen in a Tissue-Tek compound using a liquid nitrogen-cooled bath of 2-methylbutane. 7-um sections were cut using a Leica CM1860 UV cryostat, air dried for at least 30 minutes, fixed in ice-cold acetone for 10 minutes, air dried for an additional 30 minutes, and kept frozen until staining. A circle was drawn around the tissue section using a hydrophobic PAP pen, 1X PBS was added to the circle for 10 minutes, PBS was blotted off. Tissue sample was blocked by adding 5% BSA to tissue section for 1 hour at room temperature in humidified chamber or overnight at 4 degrees.

Tissue slides were washed with 1xPBS three times and stained with 1:50 GL7 FITC (clone, company), 1:100 IgD Alexa Fluor 647 (clone, company), and 1:2000 B220 Fluorochrome (clone, company) in 5% BSA at 3 hours at room temperature in humidified chamber or overnight at 4 degrees. Slides were then washed with 1xPBS three times. Mount using Prolong Gold anti-fade with Dapi (company). Lecia DM6000 Epi-Fluorescent microscope was used to capture images of whole spleens and specific germinal center sections. Leica software is LAS AF was used to process each image. GCs were manually counted from whole spleen images.

Statistical analysis

Statistical tests were performed using Prism (GraphPad) software, and p values were obtained using an unpaired one-tailed Student's t-test with a 95% confidence interval.

Publication and Contributions

This chapter is modified from the published article:

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Contributions by the individual authors are as follows:

Shirdi Schmiel – Conceptualized the project, designed/performed all experiments, gathered/analyzed all data, wrote/edited manuscript and provided partial funding for certain experiments.

Jessica Yang – Helped with the design of all experiments using the 2W-PE vaccination approach. Generated preliminary data for figures 2-4. Helped edit the manuscript.

Marc Jenkins – Consulted with designing of experiments using the 2W-PE vaccination approach. Helped edit the manuscript.

Daniel Mueller (corresponding author) – Helped design/advise experiments and concepts. Provided oversight throughout the project and funding for all projects. Wrote and edited manuscript.

Chapter 3:

Activation of A2aRs during autoimmunity

3.1 Introduction

Adenosine is an immunosuppressive purine nucleoside that promotes immunological tolerance, an active process that prevents the improper activation of autoreactive immune cells (7). Purinergic G protein-coupled receptor Adenosine 2a (A2aR) can act as a barrier to autoimmunity by limiting inflammation and negatively regulating adaptive immune responses (4). Previous studies using mouse models of T cell–mediated autoimmune disorders (12,13,14,42) have shown that A2aR signaling can restore immune homeostasis by promoting the induction of T cell anergy, regulatory T cell (Treg) differentiation, and by blocking the function of effector T cells.

Our data from chapter 2 suggest that *in vivo* A2aR signaling during the primary response to antigen plus a strong adjuvant diverts CD4 T cells away from the follicular (Tfh) and Germinal Center (GC)-Tfh lineage and can reduce humoral immune responses (73). A similar finding was recently published by Abbot and colleagues (74) where a regimen of CGS treatment following immunization with a strong adjuvant also suppressed Tfh/GC-Tfh differentiation, GC B cells, and the class-switching of B cells to IgG1 plasmablasts (74).

Although GC-Tfh cells are beneficial during the clearance of specific pathogens, they can also be detrimental if they malfunction and mistakenly provide help to pathogenic self-specific B cells. Pathological symptoms of B cell-mediated autoimmune disorders are partially attributed to enhanced production of autoantibodies (39,40,45). Full penetrance of autoantibody production requires T cell help from GC-Tfh cells. Enhanced frequencies of GC-Tfh cells has been

reported in patients suffering from antibody-mediated autoimmune disorders (43, 44). A2aRs appear to be a particularly attractive target for the treatment of B cell-dependent autoimmune disorders such as systemic lupus erythematosus (45,69) and rheumatoid arthritis (RA)(43, 44) due to their ability to divert CD4 T cells away from a GC-Tfh lineage that may provide help to pathogenic B cells.

To investigate whether A2aR signals could restore immune homeostasis during the recognition of self-antigens, we utilized a CD4 T cell transgenic mouse model of arthritis that relies on the recognition of a glucose-6-phosphate isomerase (GPI) in the context of MHC-II/I-A^{g7} by arthritic GPI-specific CD4 T cells (KRN T cells). When KRN T cells are adoptively transferred into wild type B6xB6g7 (WT)F_1 hosts, they become functionally unresponsive (anergic), and do not cause pathology (64). However, when KRN T cells transferred into a T cell deficient F_1 host (TCRα KO) that lacks peripheral immune tolerance mechanisms such as regulatory T cells (Tregs), they differentiate into effector lineages that cause pathology (64). Studies have shown that Tfh and GC-Tfh cells contribute to the formation of germinal centers, autoantibody production, and disease manifestation in the spontaneous K/BxN mouse model of arthritis (75). In these studies T cell-specific deficiencies of Tfh molecules such as the chemokine receptor type 5 (CXCR5) and signaling lymphocytic activation molecule–associated protein (SAP) were enough to protect mice from developing severe autoimmune arthritis (76). To see if activation of A2aRs can also protect against the development of autoimmune arthritis by diverting T cells away from a GC-Tfh lineage, we adoptively transferred naïve KRN T cells into WT and TCRα KO

recipients. Mice were given a daily regimen of selective A2aR agonist CGS (2.5 mg/kg i.p.) or vehicle (PBS) twice daily for the duration of the experiment.

3.2 Adenosine 2a receptor signals promote immune tolerance

KRN T cells transferred into WT mice with intact peripheral immune tolerance mechanisms did not develop arthritis regardless of PBS or CGS treatment (Fig. 8A). TCRα KO hosts that received KRN T cells and PBS treatment over the course of 10 days developed severe to moderate clinical scores (Fig. 8A) and had augmented ankle swelling (Fig. 8B). CGS treatment significantly reduced clinical scores (Fig. 8A) and ankle swelling in TCRα KO hosts (Fig 8B). Therefore, supporting previous studies that suggest A2aR signals can block the development of autoimmunity.

Autoantibodies are an important contributing factor for disease manifestation of autoimmune arthritis (75). We observed an expansion of GPI-specific GPI-specific plasmablasts ($FSC^{hi}IgH+L^{hi}B220^{lo}$ $CD38^{-}$ $GL7^{-}$) in PBS treated TCRα KO hosts, but not in WT (PBS or CGS treated) hosts (Fig. 8C). CGS treatment significantly reduced GPI-specific plasmablasts in TCRα KO hosts compared with PBS treated mice (Fig. 8C). IgG1 is the dominant isotype found in arthritic mice (75). Previous work from our lab support these findings and have observed enhanced anti-GPI IgG1 antibody titers and GPI-specific IgG1 class-switched plasmablasts in the secondary lymphoid organs (SLO) of arthritic mice (64). WT hosts had low anti-GPI IgG1 antibody titers (Fig. 1E) and only a modest number of GPI specific IgG1 class-switched plasmablasts compared with

PBS treated TCRα KO hosts (Fig. 8C, D). CGS treated TCRα KO hosts had significantly less anti-GPI IgG1 antibody titers (Fig. 8E) and GPI-specific IgG1 class-switched plasmablast numbers compared to PBS treated TCRα KO mice (Fig. 8C, D). These data suggest that A2aR-mediated suppression of autoimmune arthritis may is associated with a significant reduction of anti-GPI humoral immune responses.

3.3 A2aR signals do not promote anergy or Treg induction

Previous studies from our lab and others have reported that Tregs are indispensable for peripheral immune tolerance (8, 64). A study from our lab reported that reconstituting T cell deficient (TCRα KO) hosts with regulatory T cells (Tregs) blocked autoimmune arthritis by rendering pathogenic KRN T cells functionally unresponsive (64). Tregs co-express high levels of cell-surface ectoenzymes CD39 and CD73 that break down ATP to adenosine. Previous studies suggest that A2aR signaling promotes tolerance by inducing anergy, Treg differentiation, and enhancing Treg suppression (12). In contrast to these findings, our data in chapter 2 suggest that A2aR signals do not promote anergy or Treg induction in response to foreign antigen (73), however it remained unclear if A2aR signals responded in a similar fashion during self-antigen recognition. To further examine how A2aR signals blocked autoimmune arthritis, we first addressed the role of A2aR signals in regulating the clonal expansion of KRN T cells (Fig. 8F). KRN T cells were isolated from the secondary lymphoid organs (SLOs) ten days after KRN adoptive T cell transfer. A greater clonal expansion

of KRN T cell was seen in TCRα KO hosts (PBS and CGS treated) compared to WT hosts as previously reported (64) (Fig 8F). CGS treatment did not enhance nor reduce the expansion KRN T cells in either WT or TCRα KO hosts (Fig. 8F).

To determine if CGS treatment protected mice from severe autoimmune arthritis by promoting Treg differentiation, we examined the expression of the master regulator/transcription factor Foxp3 (Fig. 9A). CGS did not enhance the expression of Foxp3, therefore it did not promote Treg induction of KRN T cells (Fig. 9A). To determine if CGS treatment protected mice from severe autoimmune arthritis by promoting T cell anergy, we examined the expression of surface anergy molecules CD73 (*Nt5e*) and FR4 (*Izumo1r*) (63, 64). KRN T cells isolated from WT hosts expressed high levels of anergy markers CD73 and FR4 regardless of PBS or CGS treatment (Fig. 9B). Most KRN T cells from PBS treated TCRα KO mice had low expression of CD73 and FR4 (Fig. 9B). KRN T cells from CGS treated TCRα KO mice appeared to have lower CD73 and FR4 expression compare with PBS treated TCRα KO hosts (similar to our vaccine study in chapter 2). We next looked at the functional unresponsive state of KRN T cells after CGS treatment by examining the expression of the proliferative marker Ki67. To our surprise, despite reduced clinical scores, KRN T cells taken from CGS treated TCRα KO hosts expressed higher levels of the Ki67 compared with PBS treated TCRα KO hosts (Fig. 9C). We next determined if KRN T cells were functionally unresponsive by re-stimulating them *in vitro* and measuring cytokine production. To do this we isolated KRN T cells from the SLOs of different hosts and re-stimulated them *in vitro* for 4 hours using phorbol myristate acetate (PMA)

and ionomycin. Production levels of TNF-α and IL-2 were elevated in KRN T cells isolated from both CGS and PBS treated TCRα KO hosts (Fig. 9D). Cytokine production between KRN T cells isolated from CGS and PBS treated TCRα KO hosts was unchanged (Fig. 9D). Therefore, our data suggest that A2aR signals do not promote anergy or Treg differentiation.

3.4 A2aR signals divert arthritic KRN T cells away from a Tfh/GC-Tfh fate

Anergic KRN T cells from WT PBS and CGS treated hosts expressed a $CD73^{hi}$ $FR4^{hi}$ anergic phenotype, however they remained relatively low for CXCR5, a chemokine receptor highly expressed on Tfh and GC-Tfh cells. In contrast, KRN T cells isolated from PBS treated TCRα KO mice developed a sizable population of $CXCR5^{+}$ T cells that also expressed moderate levels of FR4 (data not shown) and CD73 (Fig 9E). In contrast, KRN T cells from CGS treated TCRα KO hosts had a $FR4^{lo}$ $CD73^{lo}$ $CXCR5^{-}$ profile similar to what was reported in our vaccine study (73).

To assess whether A2aR signals promoted tolerance by diverting CD4 T cells from a Tfh and GC-Tfh differentiation lineage, we looked at the expression of the cell surface markers PD-1 and the transcription factor Bcl-6 (31, 32). CD4 T cells that express the highest levels of CXCR5, Bc-l6, and PD-1 have been characterized as GC-Tfh cells ($Bcl6^{hi}$ $CXCR5^{hi}$) and are known to provide cognate help to antigen-specific B cells within germinal centers, whereas CD4 T cells expressing lower levels are characterized as Tfh cells ($Bcl6^{lo}$ $CXCR5^{lo}$) and reside at the T cell/B cell border (66). A small frequency of KRN Tfh and GC-Tfh cells

were found in PBS treated WT hosts, however, CGS treatment reduced the few Tfh and GC Tfh cells in WT treated mice (Fig. 10A, B). A sizable number of KRN Tfh and GC-Tfh cells were found in the SLOs of PBS treated TCRα KO hosts (Fig. 10A, B). CGS treatment significantly reduced the frequency and number of KRN Tfh and GC-Tfh cells in CGS treated TCRα KO hosts (Fig. 10A, B). CGS treatment also elicited a small, but significant increase in non-Tfh KRN T cells (Fig 10A, B). Lineage assessment of non-Tfh cells revealed that the number of Th1 T-bet$^+$ (Fig. 10C) and Th17 RORγt$^+$ (Fig. 10D) remained consistent between CGS and PBS treated mice.

Next, we examined whether our linage assessment matched cytokine production. A small, but significant reduction of IL-21 producing KRN T cells was observed in CGS treated TCRα KO hosts compared with PBS treated mice (Fig. 10E). IFN-γ and IL-17 production was unaffected CGS treatment (Fig. 10E). Additionally, IL-13 and IL-5 production was assessed to determine if CGS treatment affected Th2 responses. CGS treatment did not affect IL-13 or IL-5 production (data not shown).

3.5 A2aR-mediated protection is T cell-dependent

A2aR expression is highly induced on CD4 T cells following TCR ligation (9-11), however A2aRs can also be expressed on other cells of hematopoietic origin. Tfh differentiation is a multifactorial process that involves multiple cell types (31, 32, 66). Therefore, it was important to determine whether the effects of CGS were the result of direct A2aR engagement on the KRN T cells or on other

important cell types. Additionally, disease manifestation of autoimmune disorders is also multifaceted and requires not only the participation of autoreactive T cells, but also pathogenic B cells that secrete autoantibodies, and innate immune cells like neutrophils and mast cells to promote inflammatory responses (75). Figure 8 shows that CGS treatment reduced humoral immune responses; therefore, it is possible that CGS-mediated protection is due to the loss of GC-Tfh specific help to GPI-specific B cells that promotes class-switching of B cells to IgG1plasmablasts. However, it is also possible that A2aR-mediated protection is T cell independent and acting directly on the B cells or other innate immune cells.

To address this question, we generated KRN transgenic CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (KO) mice lacking A2aRs only on their T cells. KO KRN T cells and WT KRN T cells were transferred into TCRα KO recipients that lacked T cells, but globally expressed A2aRs on all other cell types. Each group was given a 10-day course of CGS or PBS treatment. In the absence of T cell-specific A2a receptors, CGS treatment failed to protect mice from autoimmune arthritis (Fig. 11). Recipients of KO KRN T cells with CGS treatment had comparable clinical scores (Fig. 11A) and ankle swelling (Fig. 11B) to that of PBS treated recipients of KO or WT KRN T cells.

In the absence of T cell specific A2aRs, CGS failed to reduce Bcl-6 expression and block GC-Tfh differentiation (Fig. 12 A, B). Likewise, CGS treatment failed to induce an increase in the frequency (Fig. 12A) and number of non-Tfh KO KRN T cell (Fig. 12B). Lineage assessment of non-Tfh cells

revealed that the frequency of Th1 Tbet$^+$ and Th17 RORγt$^+$ KRN T cells remained consistent between recipients of KO KRN and WT KRN T cells (data not shown).

To determine if A2aR-mediated reduction of humoral immune responses against GPI were also T cell-dependent we examined the frequency and number of IgG1 class-switched GPI plasmablasts in SLOs of WT and KO KRN T cell recipient mice treated with either PBS or CGS (Fig. 12C, D). In the absence of T cell specific A2aRs, CGS treatment fails to reduce the frequency (Fig. 12C) and number (Fig. 12D) of IgG1 class-switched GPI plasmablasts. Therefore, the A2aR-mediated reduction of humoral immune responses is T cell-intrinsic.

3.6 Discussion

Purinergic G protein-coupled receptor Adenosine 2a (A2aR) is thought to act as a barrier to autoimmunity by limiting inflammation (7, 70). Our data show that activation of A2aRs using a selective A2aR agonist (CGS) blocked the development severe autoimmune arthritis (Fig 8A, B). A2aR-meditated protection has been observed in other mouse models of autoimmunity (12), colitis (13), and graft-versus-host disease (14). However, our data suggest that A2aR-mediated protection was not due to induction of T cell anergy or Treg differentiation, but rather by diverting arthritic T cells away from a Tfh and GC-Tfh lineage that provides help to dangerous self-specific B cells (73).

Pathogenic autoantibodies are an important factor that contribute to the development of disease (39,40,45). IgG1 is the dominant isotype found in arthritic mice and contributes to disease manifestation by interacting with components of

the alternative complement pathway that initiates potent effector function and chemotactic activities of mast cells and neutrophils (75). Multimerized IgG1can also activate FcγRIII receptors on innate immune cells that promote downstream effector functions (75). A2aR signals significantly reduced anti-GPI IgG1 antibody titers and GPI-specific IgG1 class-switched plasmblasts (Fig. 8C, D). Our data show that CGS mediated responses are T cell dependent. In the absence of T cell specific A2aRs, CGS treatment failed to block autoimmune arthritis (Fig 11A, B), reduce GC-Tfh cells (Fig. 12 A, B), and block the differentiation of GPI-specific IgG1 class-switched plasmablasts (Fig. 12C, D). Thus, A2aR-mediated immunoregulatory responses and protection from arthritis are T cell-dependent.

Similar to our vaccine study in chapter 2, we saw a significant reduction of KRN Tfh and GC Tfh cells (Fig. 12A, B) and a slight increase of non-Tfh cells. Bcl6 is a repressor that promotes differentiation to the Tfh and GC-Tfh lineage fate, in part, by repressing other lineage-specific transcription factors such as Tbet and RORγt (31,32). However, we did not see an increase in Th1 $Tbet^+$ or Th17 $ROR\gamma t^+$ KRN T cells between CGS and PBS TCRα KO hosts (Fig. 3C-E). We also did not see an increase of Tregs ($Foxp3^+$) or Th2 ($GATA3^+$) cells in CGS treated mice (data not shown). Therefore, the increase of non-Tfh cells is comprised mainly of lineage uncommitted T cells. However, it is unclear whether these uncommitted T cells play any kind of functional role in restoring immune homeostasis.

A2aR activation reduces Tfh and GC-Tfh differentiation; however, treatment with a selective A2aR antagonist or selectively knocking-out T cell

specific A2aRs does not *enhance* Tfh or GC-Tfh cells (Fig 12 A, B). A2aR KO T cells also did not accelerate autoimmunity in PBS treated TCRα KO mice (Fig. 11A, B) nor did they induce any form of arthritis in WT hosts (data not show). It is possible that compensatory mechanisms make up for the loss of A2aR signals. A2b receptors are homologous to A2a receptors and share the same cAMP/PKA signaling pathway (16,17). A2b receptors are mainly expressed by dendritic cells and macrophages and can be expressed at low levels on lymphocytes (9,10). A2b receptors have a lower affinity for adenosine compared with A2a receptors (4). It is possible that the absence of A2a receptors may allow for available adenosine to bind and activate A2b receptors and that these signals compensate for the loss of A2aR signals. Therefore, additional studies are necessary to examine the relationship between A2a and A2b receptor signaling and their potential role in altering Tfh/GC-Tfh differentiation.

Methotrexate and sulfasalazine are popular frontline disease-modifying antirheumatic drugs (DMARD) that are commonly prescribed to individuals suffering from systemic autoimmune disorders like Rheumatoid arthritis (RA) (60). The anti-inflammatory effects of these particular DMARDS are partially attributed to augmented extracellular adenosine levels. It has been suggested that these DMARDs may act through A2a receptors. Our studies support the idea that A2aRs can alleviate autoimmune arthritis and may be an excellent therapeutic target for next-generation immune targeted therapies for individuals suffering from antibody–mediated autoimmune disorders.

3.7 Materials and Methods:

Mice

B6 (WT) mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD). KRN mice that express a TCR transgene specific for GPI/I-A^{g7} were gifts from Drs. Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA)(64). B6 TCR α-/- (B6.129S2-Tcratm1Mom/J), and $CD45.1^+$ (B6.SJL-Ptprca Pep3b/BoyJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Adora2a*$^{f/f}$ mice containing *loxP* sites on either side of exon 2 of the *Adora2a* gene (a gift from Joel Linden, La Jolla Institute for Allergy and Immunology, La Jolla, CA) (11) were crossed with CD4-Cre mice (a gift from Michael Farrar, University of Minnesota, Minneapolis, MN) to generate conditional A2aR T cell knockout (KO) mice. The breeding of $CD45.1^+$ KRN, CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out KO KRN (KO), B6 x B6.g7 (WT), TCRα-/- B6xg7, and TCRα-/- B6 x B6.g7 mice was carried out in our own colonies. All experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and the National Institutes of Health.

Adoptive Transfer of KRN T cells

Prior to adoptive transfer, WT and TCRα -/- hosts were depleted of NK cells by administering anti-asialo GM1 Ab (Wako Chemicals USA, Richmond, VA) at a dose of 25 mg/mouse via i.p. injection starting at on day 0, every 5 days

for the duration of the experiment. Donor CD45.1$^+$ KRN spleen and lymph node cells were enriched for naïve CD4 T cells using a Mouse CD4 T Cell Negative Isolation Kit (Stem Cell) per the manufacturer's instructions. Purified naïve KRN T cells were then adoptively transferred (approximately 10,000 per recipient) via tail vein injection.

Selective A2aR agonist treatment

Recipient were given a 10-12-day course of twice daily i.p. injection with the selective A2aR agonist, CGS-21680 (CGS; Tocris) 2.5 mg/kg or with vehicle alone (PBS) as previously described (12, 73).

Cell enrichment and flow cytometry

KRN T cells were analyzed by collecting the spleen and lymph node cells and staining with PE-conjugated Ab to CD45.1 (A20; eBioscience). KRN T cells were enriched using a PE positive selection kit (STEM cell) per the manufacturer's instructions. Anergy in KRN T cells was assessed by staining with CD73 (TY11.8) and folate receptor 4 (FR4, 12A5) as previously outlined (63, 64). Assessment of KRN T cell differentiation was done by surface staining enriched KRN T cells with CXCR5 (2G8), PD-1 (J43), CD4 (RM4-5), CD44 (IM7), as well as with the irrelevant cell exclusion antibodies CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8), and then fixed/permeabilized using a

fixation/permeabilization kit (eBioscience) followed by intracellular staining with Foxp3 (FJK-16s), Tbet (4B10), Bcl6 (K112-91), RORγ (Q31-378), and Ki67 (SoA15). To assess cytokine production KRN T cells were incubated for 3 h at 37°C in RPMI medium 1640 + 10% FCS in the presence of 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 1 mM ionomycin (EMD Chemicals, Gibbstown, NJ), in the final 2 hours KRN cells were incubated with 10 mg/ml brefeldin A (Sigma-Aldrich). After incubation cells were stained with the surface stained with CD4 (RM4-5), CD44 (IM7), as well as with the irrelevant cell exclusion antibodies CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8). Intracellular staining was preformed using the fixation/permeabilization kit (eBioscience) per the manufacturer's instructions and staining with IL-2 (JESS-5H4), IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), and IL-21 (clone). To assess GPI specific IgG1 plasmablasts, bulk lymphocytes were stained with B220 (RA3-6B2), GL7 (GL-7), CD38 (90), IgM (RMM-1), and IgD (11-26c.2a), as well as with the irrelevant cell exclusion antibodies CD11c (N418), CD4 (GK1.5), CD8 (53-6.7), and F4/80(BM8), and fixed/permeabilized using a fixation/permeabilization kit (eBioscience) and intracellular stained with goat anti-mouse Ig (H+L) (A11068), biotin-conjugated recombinant mouse GPI (kindly provided by Dr. Haochu Huang, University of Chicago, Chicago, IL) (64), and IgG1 (RMG1-1). All cells were analyzed using a BD LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Arthritis scoring

Ankle swelling was measured using a Quick-Mini Series 700 comparator (Mitutoyo, Aurora, IL). Changes were reported as the percent change in ankle thickness from day 0. Clinical scores to assess arthritis severity was also assigned a score from 0–3 for each paw based on the erythema/swelling as previously described (64).

Anti-GPI IgG1 Ab measurement

Serum was isolated from recipient mice on specified days and measured for anti-GPI IgG1 Abs by ELISA using recombinant mouse GPI together with IgG1-specific anti-mouse Ig reagents as previously described (64).

Statistical analysis

Statistical tests were performed using Prism (GraphPad) software, and p values were obtained using an unpaired one-tailed Student's t-test with a 95% confidence interval. Mean Arthritis Clinical Index scores were compared using the Mann–Whitney U test.

Contributions

Shirdi Schmiel – Conceptualized the project, designed/performed all experiments, gathered/analyzed all data, and provided partial funding for certain experiments.

Daniel Mueller – Helped design/advise experiments and concepts. Provided oversight throughout the project and funding for all projects.

Lokesh Kalekar –Generated data for figures 8D, and 10E.

Londyn Robinson – Generated preliminary data for Figure 8D.

Chapter 4:

A2aR therapy blocks autoimmune disease progression

4.1 Introduction

Prophylactic treatment with CGS blocks the development of severe autoimmune arthritis, however, in a clinical setting it is unlikely that patients will seek medical treatment ***prior*** to developing symptoms. For this reason, we explored the function of A2aR activation ***after*** the early onset of autoimmune arthritis. We began a CGS therapeutic regiment at a time point where KRN Tfh, GC-Tfh T cell, and anti-GPI IgG1 antibody titers are detectable.

4.2 CGS therapy blocks autoimmune arthritis disease progression

To assess the impact of activating A2aR signals ***after*** disease manifestation we adoptively transferred naïve KRN T cells into TCRα KO hosts and waited 8 days (after early signs of disease) to administer CGS (2.5 mg/kg i.p.) or vehicle alone as a control (PBS) (Fig 13). We then monitored clinical scores, collected the KRN T cells, and IgG1 class-switched GPI plasmablasts from the secondary lymphoid organs (SLOs) at days 8 (zero treatments), day 10 (4 CGS treatments), and day 12 (8 CGS treatments) (Fig 13).

Clinical symptoms of PBS treated hosts incrementally worsened over time (Fig. 14A). Activation of A2aRs significantly reduced disease severity after 4 CGS treatments (Day 10) and remained low after 8 CGS treatments (Day 12) (Fig. 14 A). Clinical scores matched the severity of ankle swelling seen in CGS and PBS treated mice (Fig. 14B). Four CGS doses significantly blocked ankle swelling of both the right and left ankles (Fig. 14B). Additional CGS doses continued to block ankle swelling at day 12 (Fig. 14B).

4.3 CGS therapy reduces GPI specific GC-Tfh cells

A previous study from our lab examined the kinetics of KRN T cell clonal expansion in arthritic TCRα KO hosts and demonstrated that the peak of expansion occurs 5 days after KRN transfer (64). A slow clonal contraction phase follows day 5 and a steady declined of KRN T cells is observed over time (64). Our data matched this observation and we see a reduction of total KRN T cells between days 8 and 10 (Fig. 15A). However, the rate of clonal contraction appears to have slowed between days 10 and 12 (Fig. 15A). A modest number of KRN Tfh and GC-Tfh cells was found in the secondary lymphoid organs (SLOs) at day 8 and continue to increase over time at days 10 and 12 (Fig 15B, C). A2aR activation beginning at day 8 reduced the number (Fig. 15D) and frequency (Fig. 15C) of GC-Tfh cells after 4 doses of CGS (Day 10). The decline of KRN GC-Tfh cell numbers continued after 8 doses of CGS at Day 12 (Fig. 15B, C). CGS therapy had a lesser effect on KRN Tfh cells where a small, but not significant reduction was observed (Fig. 15B, C). However, CGS therapy did have a small effect on KRN non-Tfh cells where a significant increase was observed in CGS treated mice (Fig. 15B, C, D). Examination of the non-Tfh cells again suggested that CGS treatment did not promote Treg induction nor did it enhance the number of Th1, Th17, or Th2 lineage cells (data not shown).

4.4 CGS therapy reduces humoral immune responses

Serum samples were collected from mice at days 8, 10, and 12 (Fig. 16A). Modest levels of anti-GPI IgG1 antibody titers were seen at day 8 and continued

to rise at days 10 and 12 in PBS treated hosts (Fig. 16A). Four CGS doses had no impact on anti-GPI IgG1 antibody titers (day 10); however, by 8 CGS doses (Day 12) the anti-GPI IgG1 antibody titers was significantly less (Fig. 16A). To our surprise, the number of IgG1 class-switched GPI plasmablasts isolated from the SLOs did not match the antibody titers observed in CGS treated mice (Fig. 16B, C). CGS treatment did not appear to reduce the overall numbers of IgG1class-switched GPI plasmablasts isolated from the SLOs (Fig. 16B, C).

4.5 CGS therapy fails to block against autoimmune arthritis in the presence of high affinity autoantibodies

Autoantibodies are an important contributing factor for disease manifestation of autoimmune arthritis with IgG1 being the dominant isotype that initiates inflammatory responses associated with disease (39,40,45). Autoantibodies contribute to disease manifestation by interacting with components of the alternative complement pathway and FcγRIII receptors that initiates potent effector functions of innate immune cells (75).

CGS therapy significantly reduced anti-GPI IgG1 antibody titers, therefore to determine if CGS mediated protection was due to the loss of high affinity antibodies we passively transferred serum from sick K/BxN mice into WT hosts (Fig. 17A). At the early signs of disease (day 6), we began administering CGS as previously described (Fig. 17A). CGS treatment failed to stop the progression of autoimmune arthritis in the presence of high affinity antibodies and immune

complexes specific for GPI, supporting the idea that CGS-mediated protection may be due, in part, to the loss of anti-GPI IgG1antibody titers (Fig. 17 B, C).

4.6 Discussion

The ultimate goal of immunotherapy is to both alleviate symptoms and restore immune homeostasis (7). Therapeutic CGS treatment not only blocked disease progression, but also reduced the presence of dangerous immunomodulatory cells that contribute to disease manifestation. Enhanced frequencies of GC-Tfh cells has been reported in patients suffering from antibody-mediated autoimmune disorders (43, 44). We saw an accumulation of autoreactive Tfh and GC-Tfh cells between days 8-12 that correlated with increasing clinical scores (Fig 14 & 15). In chapter 3, prophylactic treatment with CGS blocked the development of severe autoimmune arthritis by inhibiting KRN Tfh and GC-Tfh differentiation. We observed that eight days after KRN T cell were transferred into TCRα KO hosts, a modest frequency of Tfh and GC-Tfh cells were present in secondary lymphoid organs (SLOs)of mice (Fig. 14). CGS therapy reduced the frequency and number of GC-Tfh cells, but not Tfh cells after only 4 doses of CGS (Fig. 14). However, CGS therapy did not reduce the overall number of KRN CD4 T cells (Fig. 14A). Instead, the significant loss of GC-Tfh cells appears to be compensated by an increase of non-Tfh cells (Fig. 14). Further examination of non-Tfh cells revealed that the CGS therapy did not promote anergy or Treg induction (data not shown). Nor did it enhance or reduce the number of Th1, Th17, or Th2 cells (data not shown). Increases were among

non-committed T cells. It is unclear whether these uncommitted cells play a role in blocking autoimmune arthritis.

CGS therapy reduced anti-GPI IgG1 antibody titers after 8 doses of CGS; however, the total frequency and number of GPI IgG1 class-switched plasmablasts isolated from the SLOs organs remained the same between CGS and PBS treated hosts (Fig 16). This was a rather perplexing observation. It is possible enumerating GPI-specific IgG1 plasmablasts from only the SLOs may not accurately compute the total number of GPI-specific IgG1 antibody-secreting cells (ASC) in the host. Studies have shown that ectopic germinal centers can form in the joints of collagen-induced arthritic mice and have been found in the joints of affected patients (77). It is possible that some GPI-specific ASCs may reside in non-lymphoid organs; therefore, observations made in the SLOs may not capture the difference between CGS and PBS treated hosts.

Although discrepancies between anti-GPI IgG1 antibody titers and number of GPI IgG1 class-switched plasmablasts were observed, it was clear that CGS protection worked, in part, by reducing the levels of dangerous high affinity IgG1 antibodies responding to GPI. CGS therapy failed to provide any protection against autoimmune arthritis in the presence of transferred high affinity anti-GPI autoantibodies (Fig. 17), thus supporting the idea that significant reduction of anti-GPI IgG1 antibody titers contributes to CGS-mediated protection against disease progression. However, it is unclear whether the loss of GC-Tfh cells is important to the loss of pathogenic autoantibodies. It is possible that the loss of GC-Tfh disrupts the GC microstructure that is needed for GPI-specific B cells to

undergo extensive class-switching and affinity maturation. Additional studies are needed to examine how CGS therapy alters GC structures.

The therapeutic benefits of adenosine in autoimmune disorders have been previously reported in patients treated with methotrexate (MTX), a popular DMARD commonly prescribed to individuals suffering from systemic autoimmune disorders like rheumatoid arthritis (RA) (48). The anti-inflammatory effects of MTX are attributed to augmented levels of extracellular adenosine (60). Therapeutic CGS treatment after the development of mild arthritis blocked disease progression, reduced GC-Tfh cells, and anti-GPI IgG antibodies titers. Therefore, our data suggest that A2aR downstream signaling may serve to limit dangerous immune responses that contribute to autoimmune disease manifestations. It is of great interest to identify the factors downstream of adenosine that alleviate symptoms of arthritis. Identify potential therapeutic targets within the A2aR pathway may one day pioneer better immunotherapies for individuals suffering from autoimmune disorders.

4.7 Materials and Methods

Mice

B6 (WT) mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD). KRN mice that express a TCR transgene specific for GPI/I-A^{g7} were gifts from Drs. Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA)(64). B6 TCR α-/- (B6.129S2-Tcratm1Mom/J), and $CD45.1^+$ (B6.SJL-Ptprca Pep3b/BoyJ) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The breeding of $CD45.1^+$ KRN, B6 x B6.g7 (WT), TCRα-/- B6xg7, and TCRα-/- B6 x B6.g7 mice was carried out in our own colonies. All experimental protocols were performed in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and the National Institutes of Health.

Adoptive Transfer of KRN T cells

Donor $CD45.1^+$ KRN spleen and lymph node cells were enriched for naïve CD4 T cells using a Mouse CD4+ T Cell Negative Isolation Kit (Stem Cell) per the manufacturer's instructions. Purified naïve KRN T cells (10^4) were then adoptively transferred via tail vein injection into TCRα KO hosts.

Selective A2aR agonist treatment

Recipient were given a twice daily i.p. injection with the selective A2aR agonist, CGS-21680 (CGS; Tocris) 2.5 mg/kg or with vehicle alone (PBS) starting at day 8 post transfer (12, 73).

Serum Transfer

Serum from K/BxN mice was a gift from Dr. Bryce Binstadt. Age-matched B6 x B6.g7 (WT) mice were injected with pooled serum (200 µl, i.p.) on days 0 and 2. Clinical scores were collected every day. Therapy was administered at day 6. Mice were sacrificed on day 12.

Cell enrichment and flow cytometry

KRN T cells were analyzed by collecting the spleen and lymph node cells and staining with PE-conjugated Ab to CD45.1 (A20; eBioscience). KRN T cells were enriched using a PE positive selection kit (STEM cell) per the manufacturer's instructions. Anergy in KRN T cells was assessed by staining with CD73 (TY11.8) and folate receptor 4 (FR4, 12A5) as previously outlined (63, 64). Assessment of KRN T cell differentiation was done by surface staining enriched KRN T cells with CXCR5 (2G8), PD-1 (J43), CD4 (RM4-5), CD44 (IM7), as well as with the irrelevant cell exclusion antibodies CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8), and then fixed/permeabilized using a

fixation/permeabilization kit (eBioscience) followed by intracellular staining with Foxp3 (FJK-16s), Tbet (4B10), Bcl6 (K112-91), RORγ (Q31-378), and Ki67 (SoA15). To assess cytokine production KRN T cells were incubated for 3 h at 37°C in RPMI medium 1640 + 10% FCS in the presence of 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 1 mM ionomycin (EMD Chemicals, Gibbstown, NJ), in the final 2 hours KRN cells were incubated with 10 mg/ml brefeldin A (Sigma-Aldrich). After incubation cells were stained with the surface stained with CD4 (RM4-5), CD44 (IM7), as well as with the irrelevant cell exclusion antibodies CD11c (N418), B220 (RA3-6B2), CD8 (53-6.7), and F4/80(BM8). Intracellular staining was preformed using the fixation/permeabilization kit (eBioscience) per the manufacturer's instructions and staining with IL-2 (JESS-5H4), IFN-γ (XMG1.2), anti–TNF-α (MP6-XT22), and IL-21 (clone). To assess GPI specific IgG1 plasmablasts, bulk lymphocytes were stained with B220 (RA3-6B2), GL7 (GL-7), CD38 (90), IgM (RMM-1), and IgD (11-26c.2a), as well as with the irrelevant cell exclusion antibodies CD11c (N418), CD4 (GK1.5), CD8 (53-6.7), and F4/80(BM8), and fixed/permeabilized using a fixation/permeabilization kit (eBioscience) and intracellular stained with goat anti-mouse Ig (H+L) (A11068), biotin-conjugated recombinant mouse GPI (kindly provided by Dr. Haochu Huang, University of Chicago, Chicago, IL) (64), and IgG1 (RMG1-1). All cells were analyzed using a BD LSR II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Arthritis scoring

Ankle swelling was measured using a Quick-Mini Series 700 comparator (Mitutoyo, Aurora, IL). Changes were reported as the percent change in ankle thickness from day 0. Clinical scores to assess arthritis severity was also assigned a score from 0–3 for each paw based on the erythema/swelling as previously described (64).

Anti-GPI IgG1 Ab measurement

Serum was isolated from recipient mice on specified days and measured for anti-GPI IgG1 Abs by ELISA using recombinant mouse GPI together with IgG1-specific anti-mouse Ig reagents.

Statistical analysis

Statistical tests were performed using Prism (GraphPad) software, and p values were obtained using an unpaired one-tailed Student's t-test with a 95% confidence interval. Mean Arthritis Clinical Index scores were compared using the Mann–Whitney U test.

Contributions

Shirdi Schmiel – Conceptualized the project, designed/performed all experiments, gathered/analyzed all data, and provided partial funding for certain experiments.

Daniel Mueller – Helped design/advise experiments and concepts. Provided oversight throughout the project and funding for all projects.

Lokesh Kalekar –Generated preliminary data for Figure 16A.

Chapter 5:

Conclusions

Conclusions

The immune system is a complex circuitry of cells designed to detect and eliminate potential threats from foreign invaders while simultaneously suppressing inappropriate immune responses against self-tissues. The ultimate goal of immunotherapies is to manipulate specific components of the immune system that can resolve a loss of immune homeostasis often seen in patients suffering from cancer, autoimmune disorders, or chronic infections (7). Immunotherapy targets are often cellular and molecular components that fine-tune innate and adaptive immune responses toward a desired direction (7).

Helper CD4 T cells are critical regulators of adaptive immune responses. CD4 T cells carry out a broad-spectrum of effector functions uniquely suited to eliminate certain pathogens by activating various immune cells such as macrophages, cytotoxic CD8 T cells, and B cells (36). Identifying the factors that fine-tune CD4 T cell responses may be instrumental in the development of immune-targeted therapies and new vaccine approaches (67, 68). My thesis work has uncovered a novel role for purinergic Adenosine 2a receptor (A2a) in altering CD4 T cell responses.

Our lab was among the first to show that A2aR signals can inhibit the differentiation of germinal center T follicular helper cells (GC Tfh), a CD4 T cell subset that promotes the propagation, differentiation, and class-switching of B cells (73). The loss of GC-Tfh cells resulted in a reduction of antigen-specific humoral immune responses. However, A2aR signals did not reduce the proliferation or expansion of CD4 T cells, instead a slight shift toward another

lineage, Th17, was observed (73). Targeting A2aRs could be beneficial during mucosal infections where Th17 cells are required for pathogen clearance or during antibody-mediated autoimmune disorders where a reduction of harmful GC-Tfh cells may alleviate the severity of disease (36).

Although CD4 T cells are essential for immune homeostasis, they can at times malfunction, confuse self-antigens for foreign invaders, and mount inappropriate responses against self-tissues (46). Autoimmune disorders affect over 50 million individuals in the United States alone (47). Pathological symptoms of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are partially attributed to the enhanced production of autoantibodies (39,40,45). Full penetrance of autoantibody production requires CD4 T cell help from GC-Tfh cells. Enhanced frequency of GC-Tfh cells has been reported in patients suffering from antibody-mediated autoimmune disorders (43, 44). Studies have shown a tight correlation between the frequency of GC-Tfh cells and the onset of disease (43, 44). Our data demonstrates that A2aR signals reduced the presence of pathogenic Tfh/GC-Tfh cells, humoral immune responses against self-antigens, and blocked the development of autoimmune arthritis. Similar to our vaccine studies, our data suggests that A2aR-mediated responses and protection against arthritis are T cell-dependent.

Prophylactic treatment with CGS blocks the development of severe autoimmune arthritis, however, in a clinical setting it is unlikely that patients will seek medical treatment *before* developing symptoms. For this reason, we explored the function of A2aR activation after the early onset of disease. Therapeutic CGS

treatment of mice after developing mild arthritis blocked further disease progression and reduced pathogenic GC-Tfh cells, and harmful anti-GPI IgG1 autoantibody titers. Therefore, our data suggest that A2aR downstream signaling may serve to limit dangerous GC-Tfh cell effectors that contribute to autoimmune disease manifestations.

Understanding the mechanisms that promote and repress GC-Tfh differentiation could identify future therapeutic targets. The therapeutic benefits of adenosine in autoimmune disorders have been previously reported in patients treated with Methotrexate (MTX), a popular disease-modifying antirheumatic drug (DMARD) commonly prescribed to individuals suffering from systemic autoimmune disorders like RA (48). The anti-inflammatory effects of MTX are attributed to augmented levels of extracellular adenosine (60). My thesis research unearthed a potential T cell specific therapeutic target that may be a key regulator of adenosine-mediated therapies. Identifying potential targets within the A2aR pathway may one day pioneer better immunotherapies for individuals suffering from antibody-mediated autoimmune disorders.

Illustrations and Figures

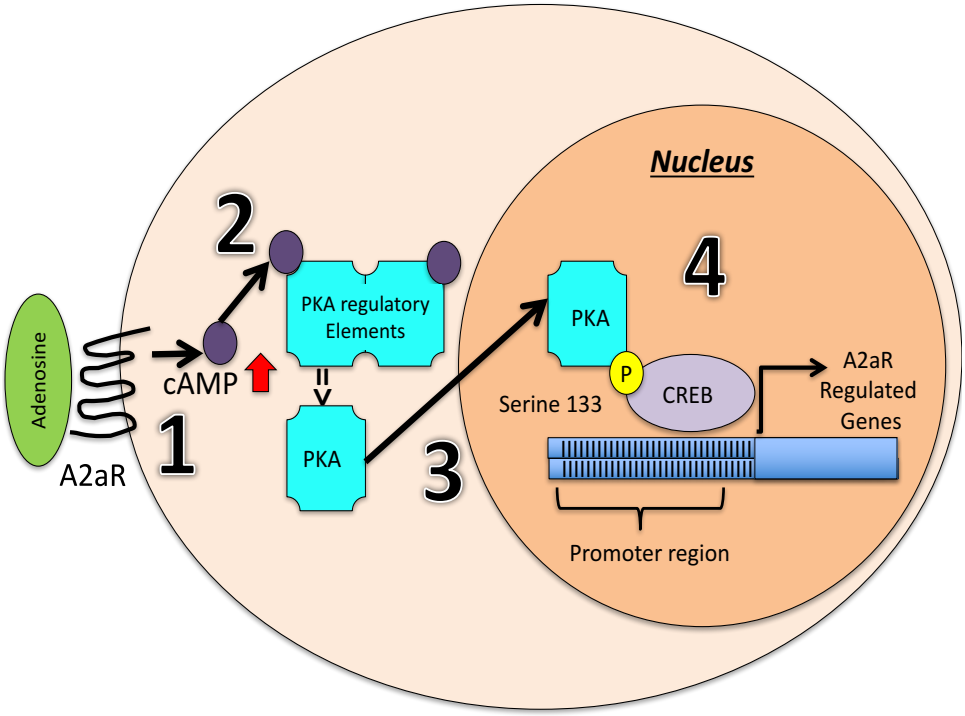

Figure 1: *A2aR signaling pathway.* (**1**) A2aR ligation promotes the accumulation of intracellular second messenger cAMP (**2**) that bind to the regulatory subunits of the Protein Kinase A (PKA) complex that then (**3**) releases the catalytic PKA subunits. (**4**) PKA can enter the nucleus and phosphorylate the Serine 133 position of various molecules including the transcription factor CREB that is often positioned at promoter site of various adenosine-mediated genes. Phosphorylation can recruit additional transcription factors to this site to either promote or repress the transcription of certain genes.

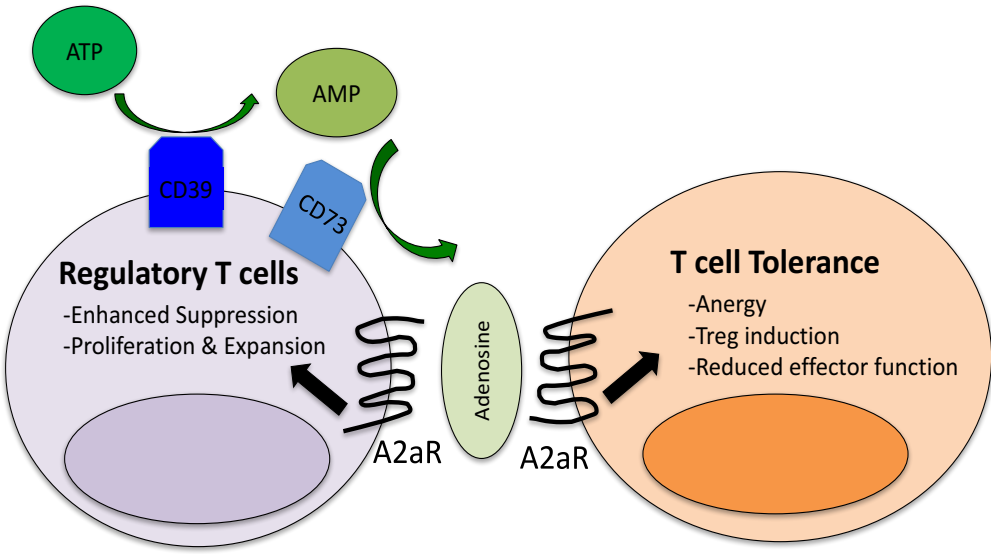

Figure 2: *A2aR signals promote T cell tolerance.* Ligation of A2a receptors by adenosine or selective agonists can promote anergy, Treg induction, enhance Treg suppression/proliferation, and reduce effector T cell functions.

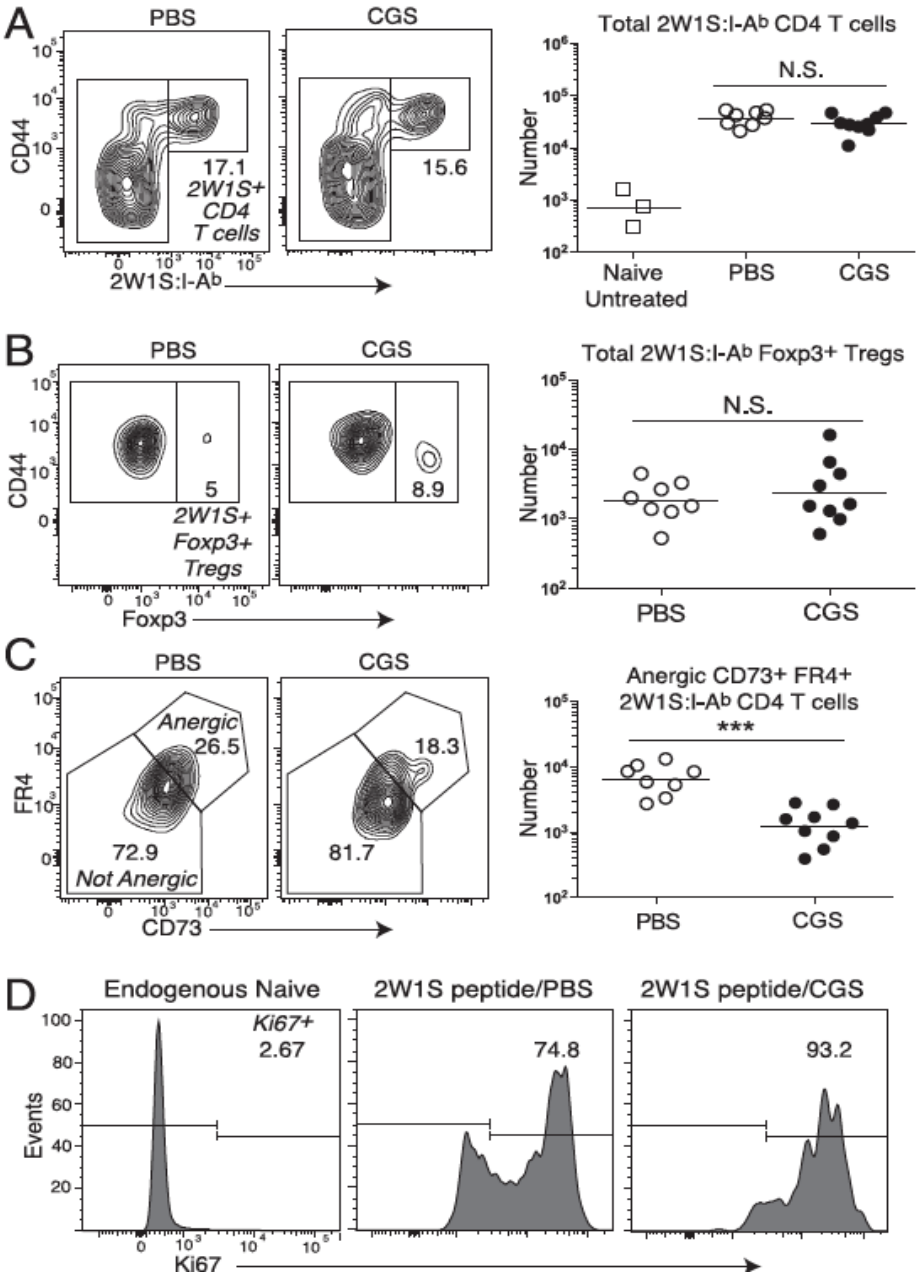

Figure 3. *A2aR signaling using the selective agonist CGS-21680 does not promote anergy or Treg induction during primary immunization*. B6 mice were immunized with 2W1S-PE and subsequently given a 7d treatment course of the selective A2aR agonist *CGS* 2.5 mg/kg or vehicle alone (*PBS*). (**A**) The frequency and number of spleen and LN 2W1S:I-A^b-specific CD4 T cells (with naive untreated mice shown for

reference). (**B**) Foxp3^{+} Tregs within the 2W1S: I-A^{b}-specific CD4 T cell compartment. (**C**) CD73^{+} FR4^{+} anergic-phenotype cells within the conventional Foxp3^{-} 2W1S:I-A^{b}-specific CD4 T cell population. (**D**) Ki67 expression in conventional Foxp3^{-} 2W1S: I-A^{b}-specific CD4 T cells 7d after 2W1S-PE immunization in the presence of CGS (*2W1S peptide/CGS*) or vehicle alone (*2W1S peptide/PBS*) (with endogenous naive polyclonal CD44^{lo} CD4 T cells shown as a control). Data are representative from three independent experiments (n = 8-9 mice per group). *P < 0.05, **P < 0.01, and ***P < 0.001

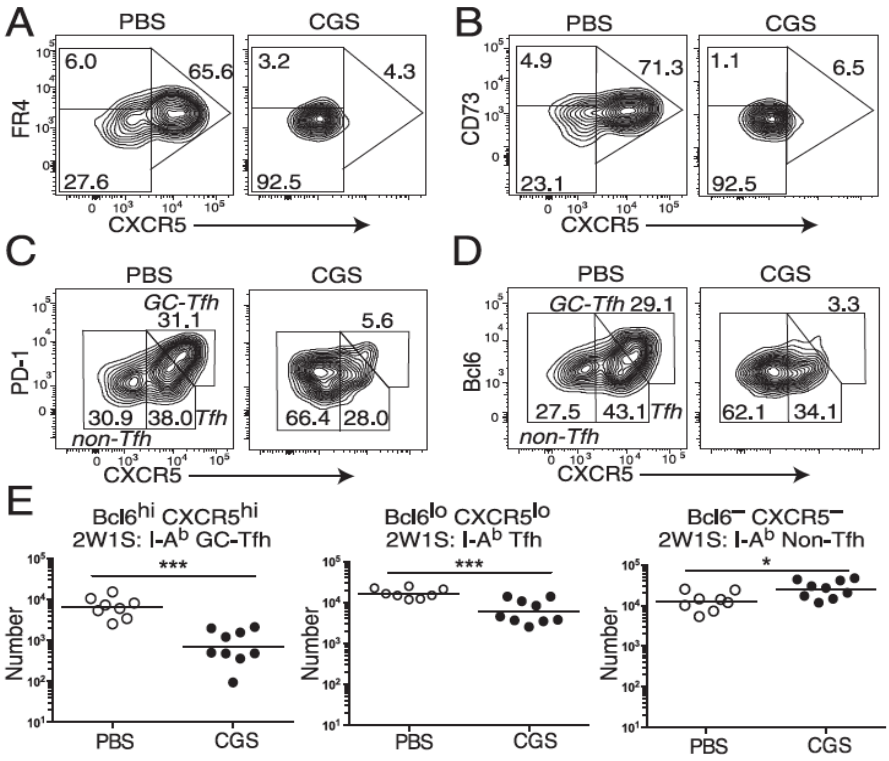

Figure 4. *A2aR activation reduces Tfh and GC-Tfh differentiation*. 2W1S:I-A^b tetramer-binding T cells were recovered from the spleen and LNs of 2W1S-PE immunized WT B6 mice after 7d of treatment with either *CGS* or vehicle alone (*PBS*). (**A**) FR4 and CXCR5, (**B**) CD73 and CXCR5, (**C**) PD-1 and CXCR5, and (**D**) Bcl6 and CXCR5 staining in 2W1S:I-A^b-specific CD4 T cells from *CGS*- or *PBS*-treated immunized mice. **(E**) Aggregate numbers of Bcl-6^{hi} CXCR5^{hi} GC-Tfh, Bcl-6^{lo} CXCR5^{lo} Tfh, and Bcl-6^{-} CXCR5^{-} non-Tfh cells that bind the 2W1S:I-A^b tetramer. Data are representative of three independent experiments (n = 8-9 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

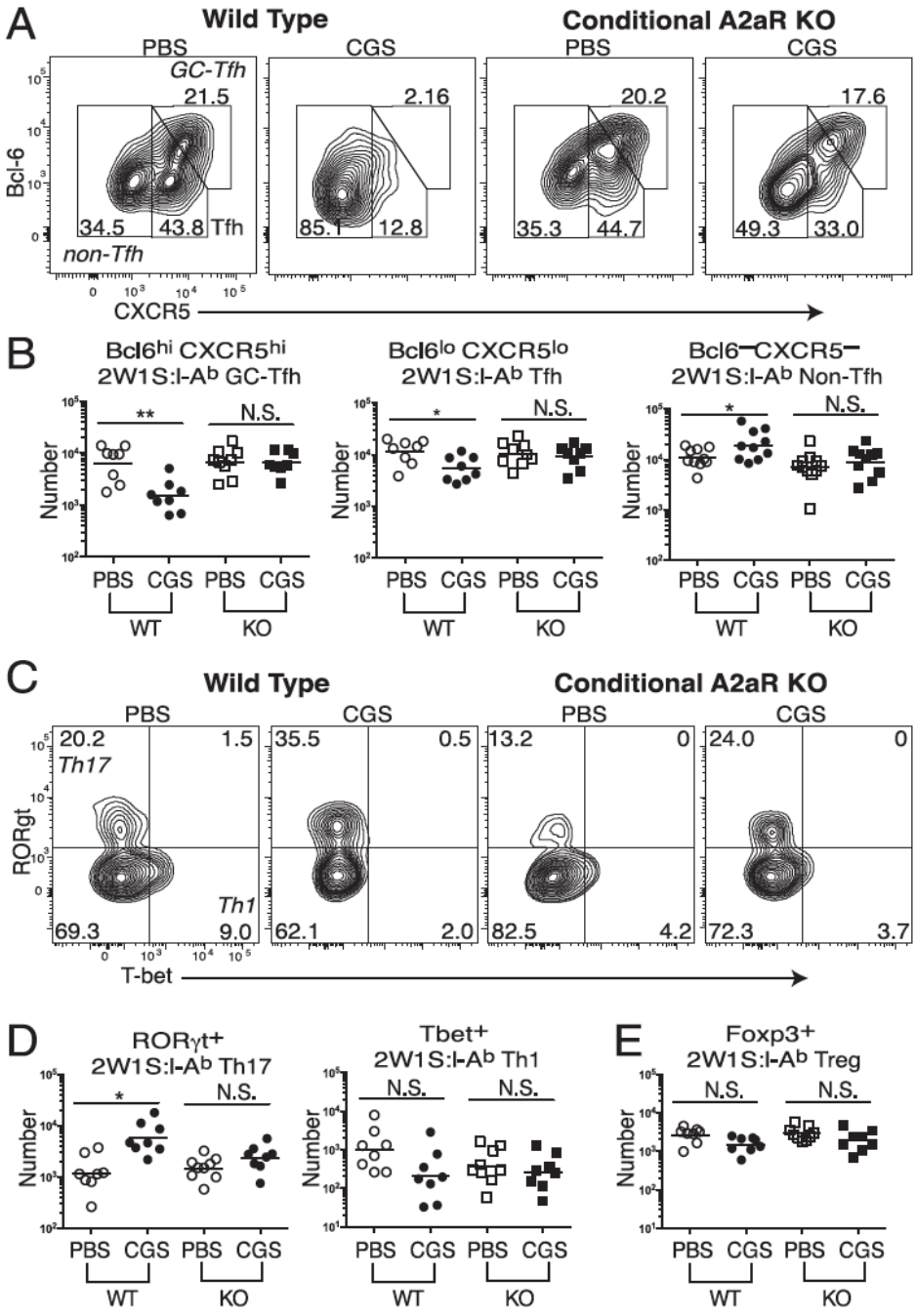

Figure 5. *A2aR inhibition of GC-Tfh differentiation is T cell intrinsic*. 2W1S: I-A^b tetramer-bound CD4 T cells were enriched from spleen and LNs of CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (*KO*) mice as well as non-Cre expressing *WT* littermates after 2W1S-PE immunization and a 7d

course of either *CGS* or *PBS* treatment. (**A**, **B**) Frequency (*A*) and number (*B*) of 2W1S-specific CD44^{hi} Foxp3^{-} CD4 T cell subsets: Bcl6^{hi} CXCR5^{hi} GC-Tfh, Bcl6^{lo} CXCR5^{lo} Tfh, and Bcl-6^{-} CXCR5^{-} non-Tfh cells. (**C, D**) Frequency (*C*) and number (*D*) of Th17 (RORγt $^{+}$ Tbet^{-}) and Th1 (RORγt $^{-}$ Tbet^{+}) lineage cells within the non-Tfh fraction of 2W1S:I-A^{b} tetramer-binding CD4 T cells. (**E**) 2W1S-specific Foxp3^{+} Treg numbers. Data are representative of three independent experiments (n = 8-9). *P < 0.05, **P < 0.01, and ***P < 0.001

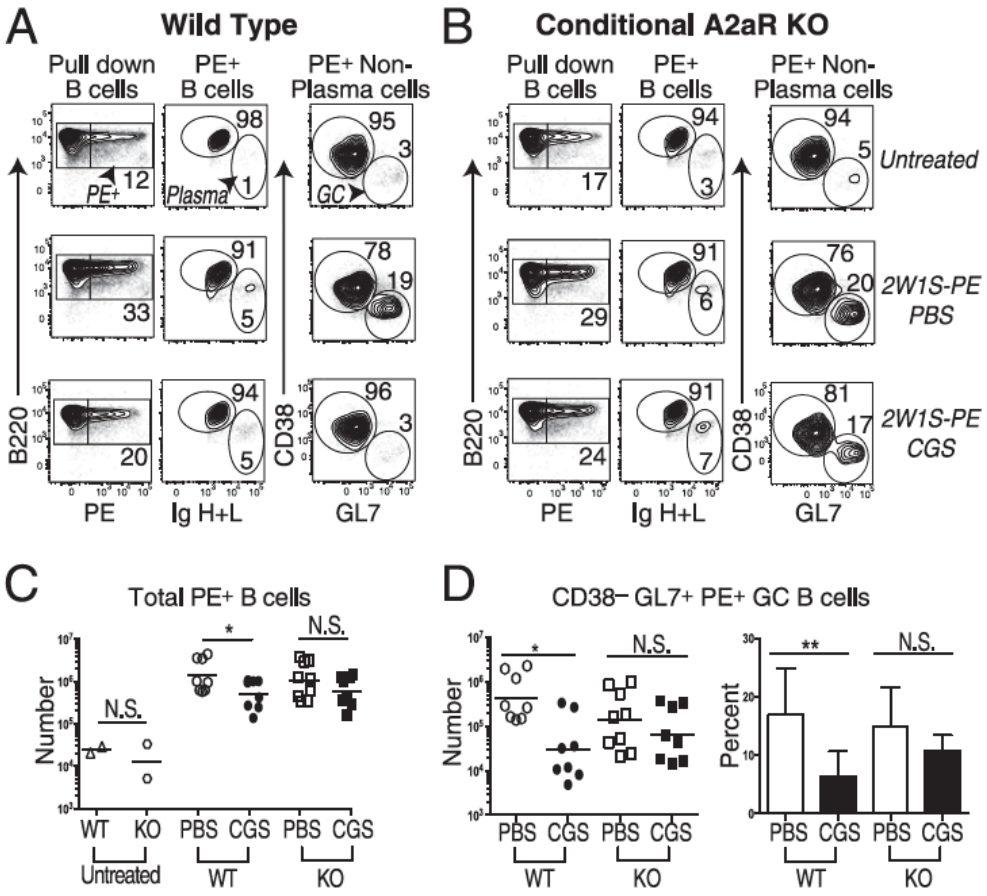

Figure 6. *T cell A2aR activation reduces GC B cell immunity*. PE-specific B cells were enriched from the spleen and LNs of 2W1S-PE primed *WT* or CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (*KO*) mice given a 7d course of *CGS* or the *PBS* vehicle alone. (**A**) Gating strategy to identify PE-specific B220^{+} total B cells (*left column*), B220^{intermediate} intracellular Ig (H+L)^{hi} plasma cells (*middle column*), as well as intracellular Ig (H+L)^{intermediate} CD38^{-} GL7^{+} GC B cells (*right column*) in control untreated (*upper row*), 2W1S-PE immunized and PBS-treated (*middle row*), and 2W1S-PE immunized and CGS-treated (*lower row*) WT mice. (**B**) Representative KO mice treated as in (*A*). (**C**) Absolute numbers of total PE-specific B cells in WT and KO mice treated as in (*A*) and (*B*), with untreated mice shown as a control. (**D**) Absolute numbers and

frequency of PE-specific polyclonal $CD38^{-}$ $GL7^{+}$ GC B cells in immunized WT and KO mice treated as in (*A*) and (*B*). Data are representative of three independent experiments (n = 8-9 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

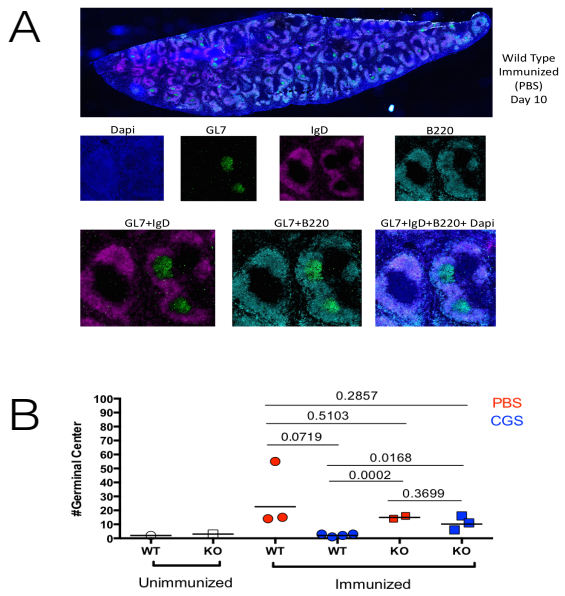

Figure 7. *T cell A2aR activation reduces the quantity of Germinal Center.* Tissue slides of spleens from 2W1S-PE primed *WT* or CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (*KO*) mice given a 7d course of *CGS* or the *PBS* vehicle alone. (**A**) Image of a whole spleen from a 2W1S-PE primed *WT* mouse given a 7d course of *PBS.* Middle panels are focused on a single germinal center from the spleen of an immunized WT mouse given PBS. Each panel shows four individual staining fluorchromes for Dapi, IgD, GL7, and B220 that help distinguish GCs. The bottom panel overlaps the IgD, GL7 to clearly distinguish GCs, it also overlaps GL7 and B220 to show that cells in the GC complex were true B cells. Finally, the bottom right panel shows the overlap of all three fluorchromes that include Dapi to show the general requirements to be considered a GC. **(B)** based on the criteria from figure A, we manually counted the number of GC present in unimmunized, immunized, CGS or PBS treated WT and KO mice. Data are representative of two independent experiments (n = 1-4 mice).

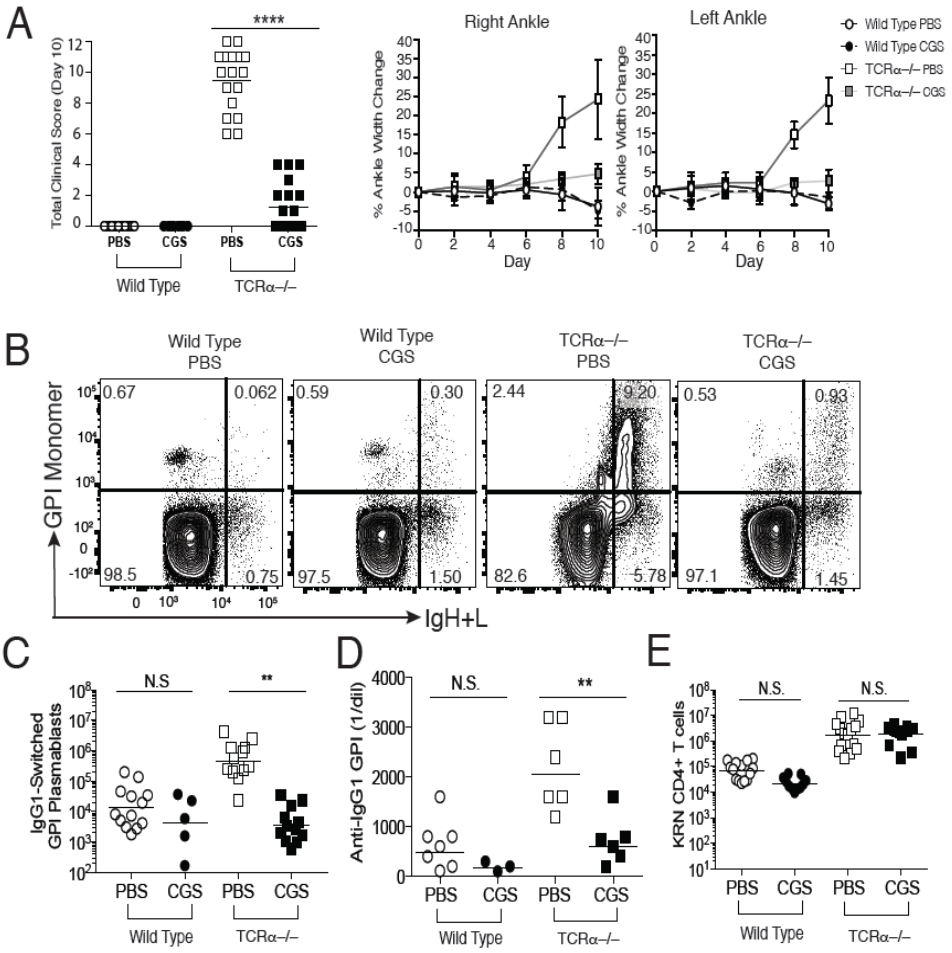

Figure 8: *A2aR signals block autoimmune arthritis.* Naïve CD45.1^{+} KRN T cells (10^{4}) were adoptively transferred into WT and TCRα KO hosts. After 24 hours, mice were injected (i.p.) twice daily with a selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (10 days). (**A**) Clinical disease scores taken at day 10 (*left*) and mean percent changes of ankle swelling/size (mm) over the duration of the experiment (*right*). (**B**) Bulk B cells were stained for intracellular Ig H+L chain accumulation and GPI-binding capacity to examine GPI-specific plasmablasts (**C**) B220^{intermediate} intracellular Ig (H+L) ^{hi} GL7^{-} CD38^{-}IgG1^{+}GPI specific plasmablasts were enumerated. (**D**) Anti-GPI IgG1 titers measured from serum of mice at day 10 via ELISA. (**E**) Total

number of CD45.1$^+$ KRN T cells isolated from the spleen and lymph nodes of WT and TCRα KO (CGS and PBS treated hosts) were enumerated. Data are representative of three-five independent experiments (n = 6-17). *P < 0.05, **P < 0.01, and ***P < 0.001

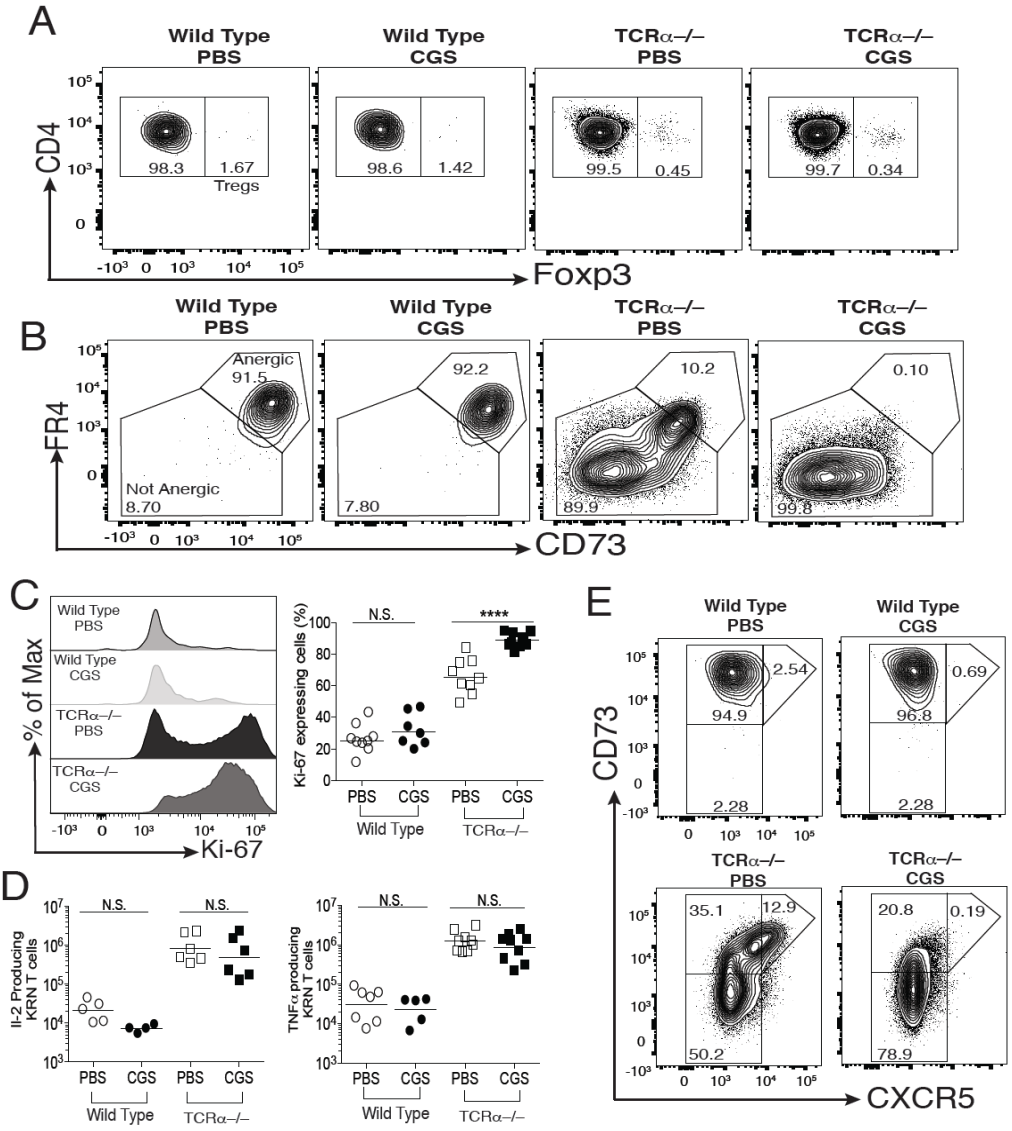

Figure 9: *A2aR signaling using the selective agonist CGS-21680 does not promote anergy or Treg induction.* Naïve CD45.1^{+} KRN T cells (10^{4}) were adoptively transferred into WT and TCRα KO hosts. After 24 hours, mice were injected (i.p.) twice daily with selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (10 days). KRN T cells were isolated from the spleen and lymph nodes of PBS and CGS treated WT

and TCRα KO hosts at day 10. (**A**) The frequency of KRN Foxp3$^+$ Tregs. (**B**) CD73$^+$ FR4$^+$ anergic-phenotype cells within the conventional Foxp3$^-$ KRN T cell population. (**C**) Ki67 expression within conventional Foxp3$^-$ KRN T cells. (**D**) KRN T cell production of IL-2 and TNF-α after PMA/ionomycin re-stimulation. (**E**) CD73$^+$ and CXCR5$^+$ KRN T cells. Data are representative from three independent experiments (n = 5-10 mice per group). *P < 0.05, **P < 0.01, and ***P < 0.001

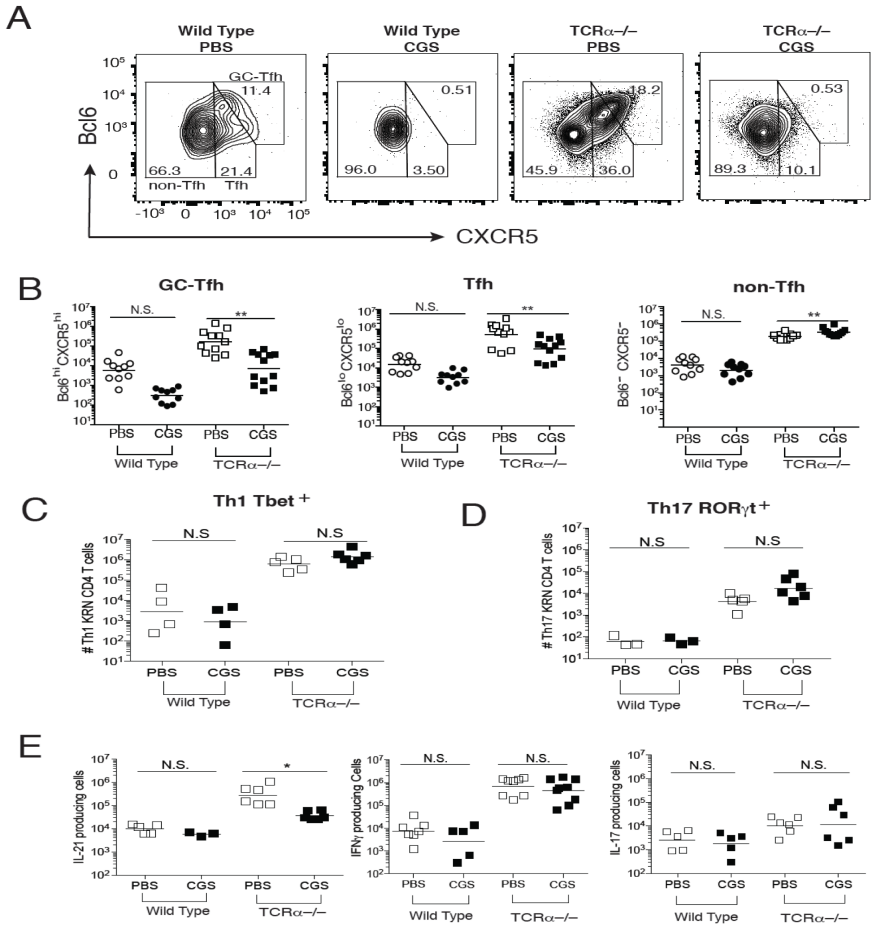

Figure 10: *A2aR activation reduces Tfh and GC-Tfh differentiation*. KRN T cells were isolated from the spleen and lymph nodes of PBS and CGS treated WT and TCRα KO hosts at day 10. (**A**) Bcl6 and CXCR5 staining of KRN T cells from CGS- or PBS-treated hosts. **(B)** Aggregate numbers of Bcl-6^{hi} CXCR5^{hi} (GC-Tfh), Bcl-6^{lo} CXCR5^{lo} (Tfh), and Bcl-6^{-} CXCR5^{-} (non-Tfh) KRN T cells. (**C**) Number Th1 (Tbet^{+}) and (**D**) Th17 (RORγ^{+}) lineage cells within the non-Tfh fraction of KRN T cells. (**E**) KRN T cell production of IL-

21, IFN-γ, and IL-17 after PMA/ionomycin re-stimulation. Data are representative of three-four independent experiments (n = 4-12 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

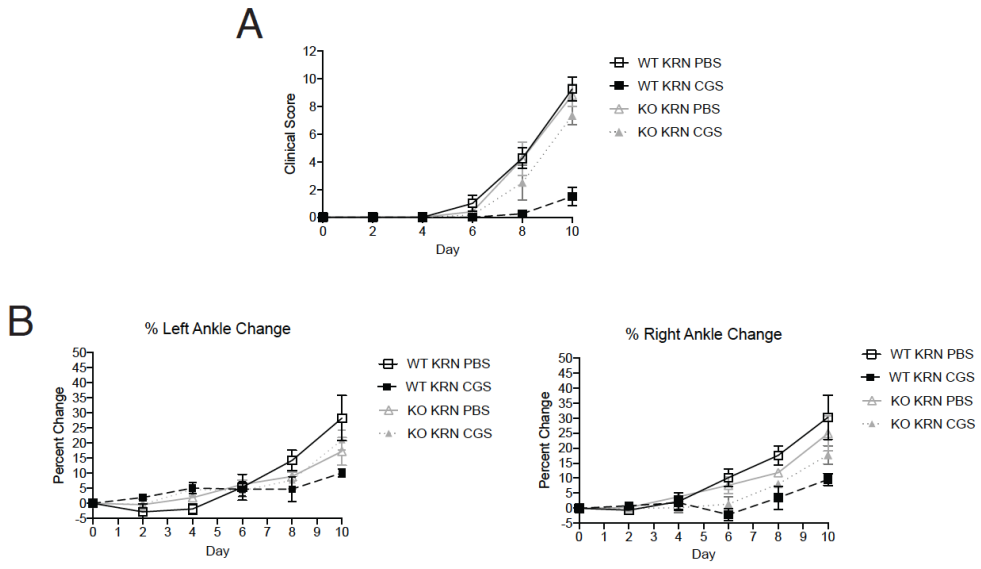

Figure 11: *CGS-mediated protection is T cell-dependent.* Naïve CD45.1 KRN+ T cells (10^4) were enriched from spleen and LNs of KRN CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (KO) mice and (WT) KRN mice. After 24 hours, mice were injected (i.p.) twice daily with selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (10 days). (**A**) Mean clinical disease scores over time and (**B**) percent change of ankle swelling/size (mm) over the duration of the experiment. Data are representative of three-four independent experiments (n = 4-12 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

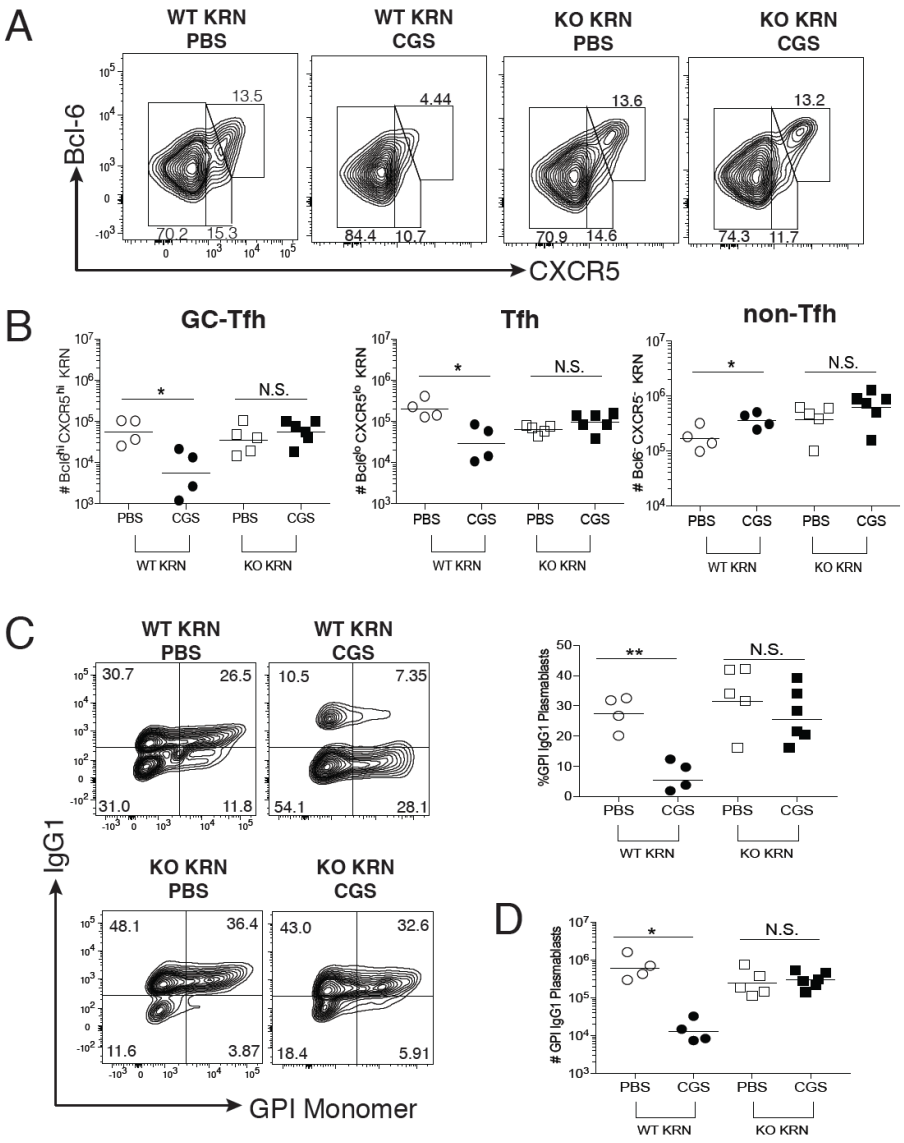

Figure 12: *A2aR inhibition of GC-Tfh differentiation and humoral immune responses are T cell dependent*. Naïve KRN CD4-Cre *Adora2a*$^{f/f}$ conditional knock-out (KO) T cells (10^4) and WT KRN T cells were transferred into TCRα KO hosts. After 24 hours, mice were injected (i.p.) twice daily with selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (10 days). (**A**, **B**) Frequency (*A*) and number (*B*) of KRN CD44^{hi} Foxp3^{-} CD4 T cell subsets: Bcl6^{hi} CXCR5^{hi} (GC-Tfh), Bcl6^{lo} CXCR5^{lo} (Tfh), and Bcl-6^{-} CXCR5^{-} (non-Tfh cells). (**C**) Frequency

and number (**D)** of $B220^{intermediate}$ intracellular Ig $(H+L)^{hi}$ $GL7^{-}$ $CD38^{-}$ $IgG1^{+}$GPI specific plasmablasts. Data are representative of three independent experiments (n = 4-7). *P < 0.05, **P < 0.01, and ***P < 0.001

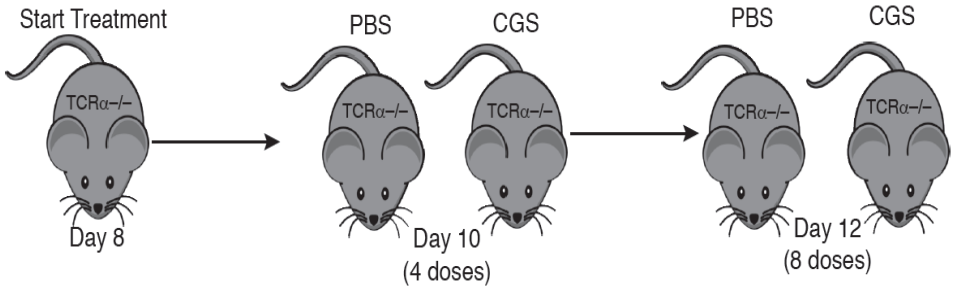

Figure 13: *Experimental set up for CGS therapy.* Naïve CD45.1^{+} KRN T cells (10^{4}) were adoptively transferred into WT and TCRα KO hosts. After 8 days, mice were injected (i.p.) twice daily with selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (12 days). Serum from blood samples and KRN T cells from the spleen and lymph nodes of PBS and CGS treated WT and TCRα KO hosts were collected at days 8, 10, 12.

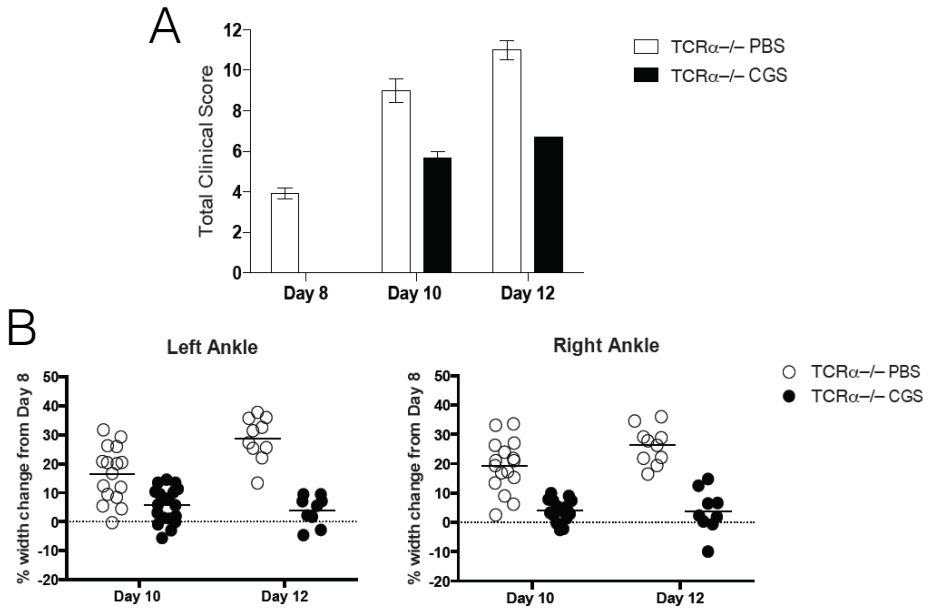

Figure 14: *Activation of A2aRs blocks progression of autoimmune arthritis.* (**A**) Clinical disease scores observed at days 8, 10, and 12. (**B**) Percent change of ankle swelling/size (mm) from day 8. Data are representative of three-four independent experiments (n = 4-12 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

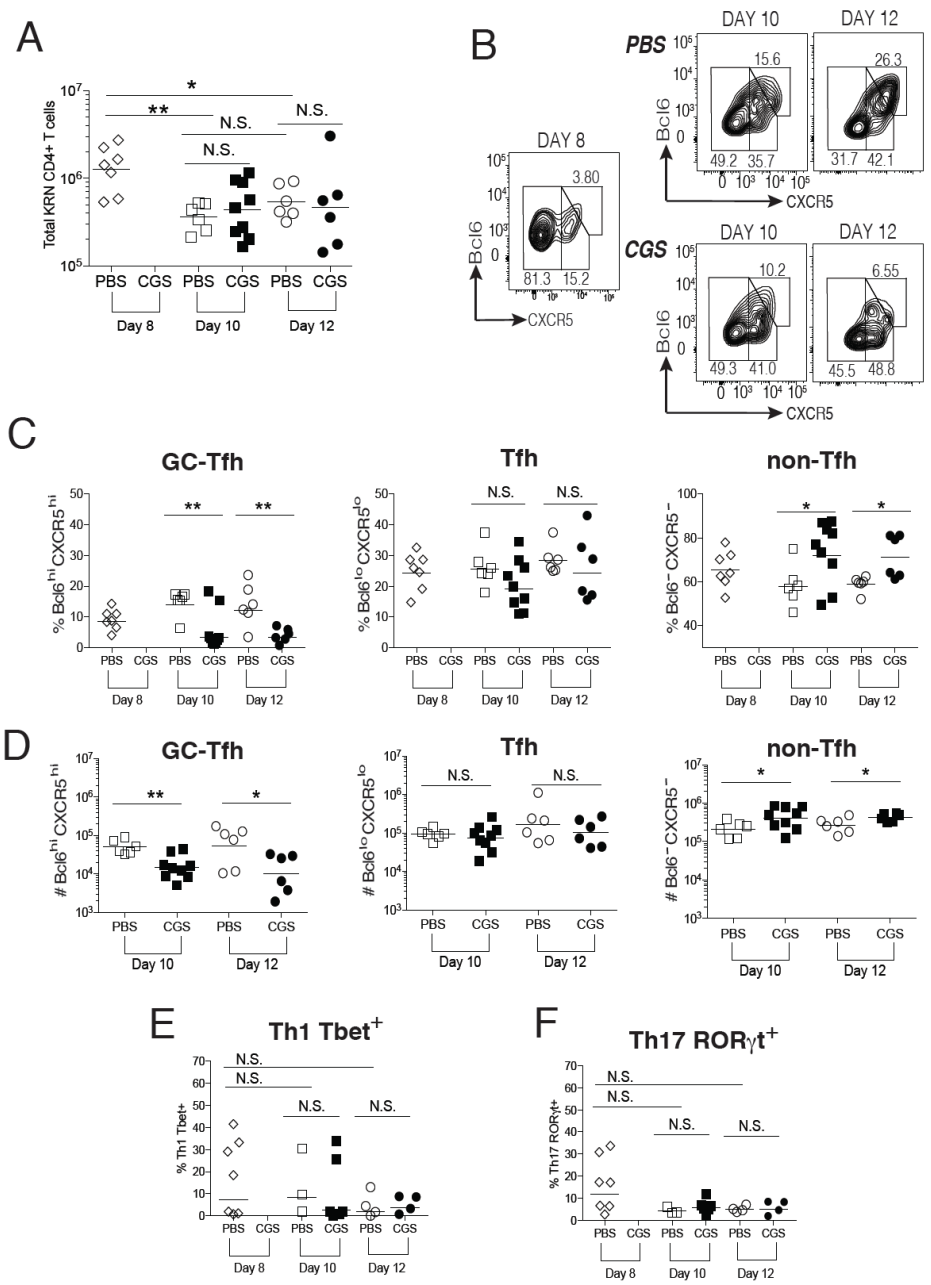

Figure 15: *CGS therapy reduces KRN GC-Tfh cells*. KRN T cells were isolated from the spleen and lymph nodes of PBS and CGS treated TCRα

KO hosts at days 8, 10, and 12. (**A**) The total number of CD45.1^{+} KRN T cells isolated from the spleen and lymph nodes of CGS or PBS TCRα KO hosts. (**B**) Bcl6 and CXCR5 staining in KRN T cells from isolated from CGS- or PBS-treated mice. **(C)** Percent and (**D**) number of Bcl-6^{hi} CXCR5^{hi} (GC-Tfh), Bcl-6^{lo} CXCR5^{lo} (Tfh), and Bcl-6$^{–}$ CXCR5$^{–}$ (non-Tfh) KRN T cells isolated at days 8, 10, and 12. Frequency of non-Tfh (**E**) Th1 Tbet^{+} and (**F**) Th17 RORγt^{+}. Data are representative of three independent experiments (n = 8-12 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

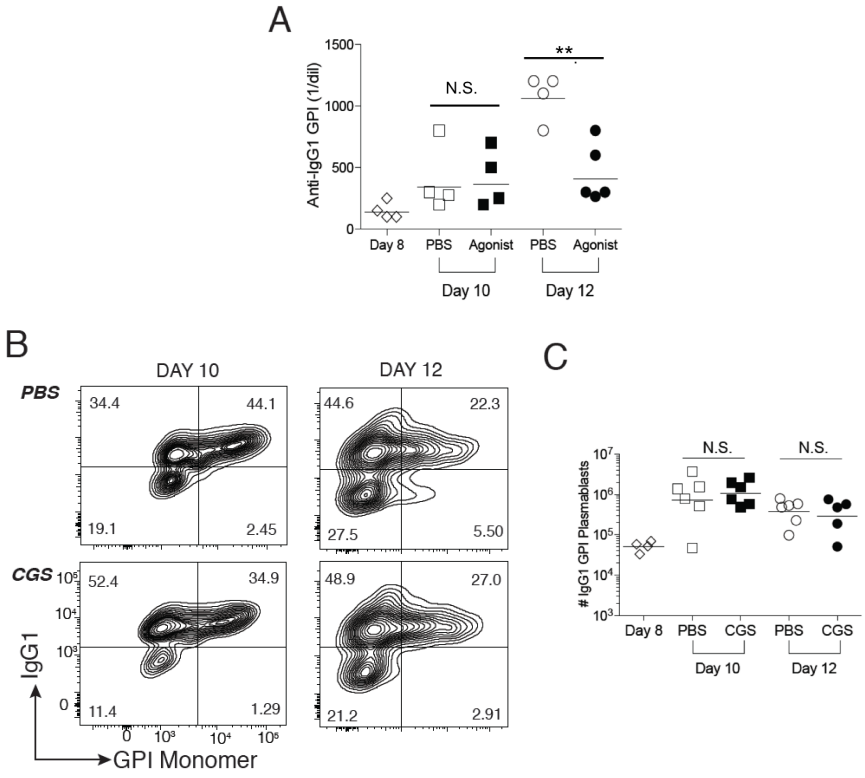

Figure 16: *A2aR activation reduced anti-GPI IgG1 antibody titers, but not GPI-specific IgG1 class-switched plasmablasts.* Serum from CGS and PBS treated mice at days 8, 10, and 12 was also collected. (**A**) Anti-GPI IgG1 titers measured from the serum of mice at day 8, 10, and 12 via ELISA. Bulk B cells from the spleen and lymph nodes of CGS and PBS treated mice at days 8, 10, and 12 were isolated, stained, and examined (**B**) Frequency and (**C**) number of $B220^{intermediate}$ intracellular Ig $(H+L)^{hi}$ $GL7^{-}$ $CD38^{-}IgG1^{+}$GPI specific plasmablasts from the spleen and lymph nodes of CGS and PBS treated mice at days 8, 10, and 12. Data are representative of three independent experiments (n = 4-7). *P < 0.05, **P < 0.01, and ***P < 0.001

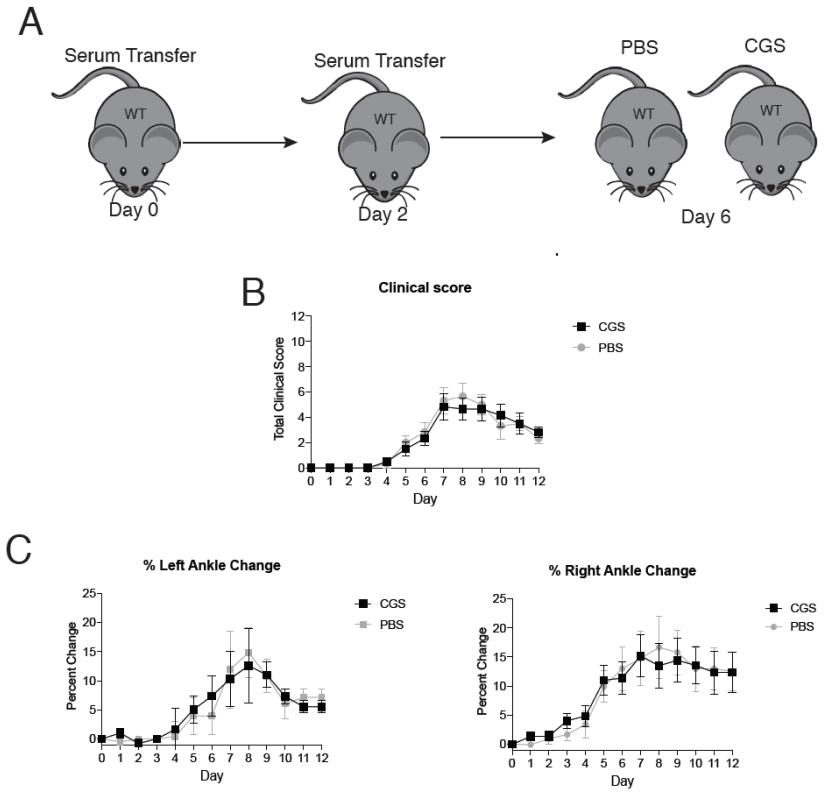

Figure 17: *CGS therapy fails to stop disease progression in the presence of high affinity antibodies.* Serum collected from arthritic K/BxN mice was i.p. injected into WT mice at Days 0 and 2. Six days after serum transfer mice were injected (i.p.) twice daily with selective A2aR agonist CGS (2.5 mg/kg) or vehicle alone (PBS) for the duration of the experiment (12 days). (**A**) Mean clinical disease scores observed at days 8, 10, and 12. (**B**) Mean percent change of ankle swelling/size (mm). Data are representative of three independent experiments (n = 8-10 mice). *P < 0.05, **P < 0.01, and ***P < 0.001

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